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Combination Therapy With Aerosol Il-2 And Nk Cells For The Treatment Of Osteosarcoma Lung Metastasis

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COMBINATION THERAPY WITH AEROSOL IL-2 AND NK CELLS FOR THE TREATMENT OF OSTEOSARCOMA LUNG METASTASIS

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A

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by

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DEDICATION

To my mother, Ana Teresa De la Vega. All the success I've had in my life I owe it to her and her guidance. I love you, Mom.

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I am fortunate to have been raised in a family that fosters education and learning. My mother Ana Teresa De la Vega has been an inspiration to me and all the success I have I owe it to her. Thank you, Mom.

COMBINATION THERAPY WITH AEROSOL IL-2 AND NK CELLS FOR THE TREATMENT OF OSTEOSARCOMA LUNG METASTASIS

Sergei R. Guma De la Vega, BS

Supervisory Professor: Eugenie S. Kleinerman, MD

The survival of patients with osteosarcoma lung metastases has not improved in the last 20 years. Novel alternative therapies are needed. The purpose of this investigation was to evaluate the efficacy of combining natural killer [NK] cell therapy with aerosol interleukin-2 [IL-2] for the treatment of osteosarcoma lung metastasis. The expression of NKG2D ligands was analyzed in five different human osteosarcoma cell lines and 103 patient samples (47 from primary tumors and 56 from lung metastases). We discovered that osteosarcoma expresses the ligands for the NKG2D. *In vitro* studies demonstrated that NK-mediated killing of osteosarcoma cells is dependent on the NKG2D-NKG2D ligand interaction and that cytotoxicity correlates with the level of NKG2D ligand expression. Aerosol IL-2 increased NK cell numbers in the lung but not in other organs. This increase in NK numbers was due to an aerosol IL-2-induced increase in NK proliferation that occurs almost exclusively in the lung. Aerosol IL-2 also increased the NK cell infiltration within the lung metastatic nodules. Compared to control, NK cell treatment alone and aerosol IL-2 treatment alone, aerosol IL-2 + NK cell therapy had a higher therapeutic efficacy, as judged by a greater decrease in the number and sizes of tumors nodules, as well as by a greater increase in tumor apoptosis. Furthermore, aerosol IL-2 + NK cell therapy significantly improved the

survival of mice with osteosarcoma lung metastasis. In addition, there was no evidence of IL-2-associated systemic toxicities.

In conclusion, combining NK cell therapy with aerosol IL-2 achieved organ-specific NK cell migration and proliferation in the lung and reduced pulmonary metastatic burden, while at the same time avoided the systemic toxicities associated with IL-2. We believe that a combination therapy consisting of aerosol IL-2 with NK cell infusions can be a new and effective therapeutic approach for the treatment of patients with osteosarcoma lung metastasis.

Table of Contents

LIST OF FIGURES

INTRODUCTION

Chapter 1:

Introduction: background, rationale and research plan

Osteosarcoma

Osteosarcoma is the most common primary bone cancer in pediatric patients. It is the second most common primary malignant bone tumor in the general population, after multiple myeloma. 75% of all osteosarcoma cases occur between the ages of 15 and 25 years [1]. It is the fifth most common primary malignant tumor in adolescence. The most common presenting symptom is pain at the extremities, usually accompanied by a palpable mass upon physical examination [2].

The treatment of osteosarcoma originally consisted of surgery with or without radiation therapy. Overall 2-year survival rates were 15 – 20%. In most cases, microscopic metastases are present in the lung at the time of diagnosis. For this reason, osteosarcoma that was only treated with surgery and/or radiation had a metastatic recurrence of 80% [3]. With the advent of neoadjuvant and postoperative chemotherapy for primary osteosarcoma treatment, 5 year survival rates have increased to 68% [4]. The current standard of care consists of surgery and a combination of high-dose methotrexate, cisplatin, adriamycin and ifosfamide [2]. However, prognosis for patients with pulmonary metastasis is dismal, with a 5 year survival of 33% [5].

The most frequent site of osteosarcoma metastasis is the lung (81.2%), followed by the bone (34.2%) [5]. Most deaths are due to respiratory failure caused by metastatic burden [2]. The current standard treatment for osteosarcoma lung metastasis consists of surgical resection with preoperative and postoperative chemotherapy. Chemotherapy consists of cisplatin,

doxorubicin, and high-dose methotrexate with leucovorin. The survival for relapsed patients is very low, with less than 20 % long term post-relapse survival. Treatment for recurrent lung metastasis is complete surgical removal of all metastatic foci. The role of chemotherapy for recurrent osteosarcoma is much less defined, with no accepted standard therapy [6]. It is imperative to discover and develop new alternative therapies for primary and recurrent osteosarcoma lung metastasis.

Immunotherapy for osteosarcoma

Our laboratory has had success with the use of immunotherapy for the treatment of osteosarcoma in pre-clinical and clinical settings. We demonstrated that liposomal muramyl tripeptide [L-MTP-PE] activates the tumoricidal properties of blood monocytes [7]. A Phase II trial demonstrated that L-MTP-PE significantly increased the progression-free interval and the long-term survival of patients with relapsed pulmonary osteosarcoma metastasis [8]. A Phase III trial demonstrated that the addition of L-MTP-PE to standard chemotherapy increased the 6-year survival of pediatric patients with primary osteosarcoma from 70 to 78% [9]. L-MTP-PE has been approved for the treatment of high-grade resectable nonmetastatic osteosarcoma in the European Union. It has been the first new agent approved for the treatment of osteosarcoma in the last 20 years [10].

We have also demonstrated the effectiveness of using genetically modified T cells against osteosarcoma lung metastasis in a preclinical model. We

found that IL-11Rα is highly expressed in patient specimens of osteosarcoma lung metastasis. T cells were genetically modified to express a chimeric antigen receptor against IL-11Rα. Treatment with IL-11Rα-CAR-specific T cells significantly reduced pulmonary osteosarcoma metastasis in an athymic mouse model. [11]. The success of these immunotherapies supports the investigation of other immune-based therapies, such as adoptive natural killer [NK] cell therapy.

Natural killer cells

Human NK cells comprise about 10 – 15% of peripheral lymphocytes [12]. They are considered part of the innate immune system due to their ability to detect and kill virus infected and tumor cells without prior sensitization [13]. They play an essential role in immunosurveillance. Patients with lower peripheral natural cytotoxicity have greater cancer predisposition [14]. Patients with NK cell deficiencies are more susceptible to viral infection, especially from members of the Herpesviridae family [15]. In addition to their cytotoxicity, activated NK cells also secrete a variety of cytokines, including INFɣ, TNFα, IL-12, GM-CSF and MIP-1αβ, which can activate other members of the immune system [16]. They themselves can be stimulated by IL-12, IL-15, IL-18, IL-21 and IL-2 [17].

The recognition of a target cell by NK cells is a complex interplay between inhibitory and activating NK receptors and their respective ligands. CD94/NKG2A heterodimers and the killer-cell immunoglobulin-like receptors (KIRs) are the major inhibitory receptors in human NK cells. KIRs play a major role in NK cell

self-tolerance [18]. They recognize specific HLA-B and HLA-C allotypes, while CD94/NKG2A heterodimers bind to HLA-E [19]. Loss of HLA is a frequent adaptive mechanism used by cancer cells to escape cytotoxic T cell recognition. However, this same mechanism renders them more susceptible to NK cytotoxicity [20]. In humans, the major activating NK receptors are NKG2D, D-NAM1, and the natural cytotoxicity receptors (NCRs) [21]. The NCRs, which consist of NKp46, NKp44 and NKp30, recognize transmembrane-anchored polypeptides with immune tyrosine-based activating motifs (ITAM). The specific ligands for the NCRs are currently unknown, but current data suggest that normal tissues either do not express these ligands or that they express very low levels of them [22]. D-NAM1 recognizes poliovirus receptor and nectin-2, both members of the nectin family. D-NAM1 ligands are expressed in a variety of human cancers. However they are also ubiquitous on normal tissue. This suggests that NK activation by D-NAM1 is highly dependent on the HLA status [22]. NKG2D is the best characterized NK activating receptor. It binds and recognizes MIC A/B and the ULBP family members. NKG2D ligands are generally absent from normal cells. MIC A/B is only expressed on GI epithelial cells on healthy individuals. This is believed to be due to epithelial contact with intestinal microbes. NKG2D ligands are upregulated under conditions of cellular stress, including viral infection, classical heat shock and malignant transformation [23]. Therefore, NKG2D ligands tend to be expressed on a variety of human cancers [24-27]. Activation signals generated by NKG2D-ligand binding can bypass signals

generated by NK inhibitory receptors [23], rendering NK-mediated killing of cancer cells possible even in the presence of HLA expression.

NKG2D recognition of its ligand activates NK killing through the secretion of cytotoxic granules into the intercellular space. Major granule proteins include perforin, which disrupts the cellular membrane, and the granzymes, serine proteases that trigger the apoptotic pathway. NK cells also express TRAIL and FasL, both of which can trigger apoptosis by binding to their corresponding receptors [28]. In addition to NK killing, target recognition also triggers NK cytokine production [29]. This further contributes to the overall immune response. For example, activated NK cells secrete INFɣ and MIP-1α, potent macrophage activators [17].

NK cell cancer immunotherapy

In 2002, Ruggeri *et al* [30] demonstrated that the survival of AML patients treated with T cell-depleted allogeneic hematopoietic transplantation was significantly increased in the presence of KIR ligand incompatibility form donor to recipient. KIR ligand incompatibility increased 5 year survival from 5% to 60%. It also prevented the appearance of graft-versus-host disease. Increased 5 year survival (from 48% to 87%) was subsequently observed in CML and MDS patients treated with T cell depleted allogeneic transplantation in the presence of KIR mismatch [31]. This drastic increase in survival and the leading role NK cells played in the eradication of said malignancies has increased interest in the use of

NK cells as potential new anti-cancer therapeutics. NK cell associated graft-vs-tumor effects have also been reported in patients with pediatric solid tumors treated with haploidentical stem cell transplantation [32, 33].

A number of phase I clinical trials have been performed to evaluate the safety of allogeneic natural killer cell transplantation as treatment for AML. These have demonstrated successful engraftment of the donor NK cells, with little to no side-effects and with evidence of clinical response [34-36]. However, these trials required the administration of intravenous IL-2 to maintain *in vivo* NK expansion.

One of the limiting factors of NK cell therapy is the ability to grow them in sufficient numbers. The highest dose of NK cells that have been infused into patients was generated through *ex vivo* expansion [37]. Several different methods have been developed for *ex vivo* NK cell expansion. The uses of both soluble and membrane-bound IL-15, as well as other cytokines, such as IL-2 and IL-7, have been studied [38]. Denman *et al* established an *ex vivo* method to expand donor NK cells using IL-2 and a genetically modified K562 artificial antigen presenting cell [aAPC] expressing membrane bound IL-21[39]. This method promoted a sustained mean 47,967-fold NK expansion after 42 days, as well as increasing NK cytotoxicity and NKG2D expression. In addition, membrane bound IL-21 promoted a significant increase in NK telomere length and greatly reduced senescence. In contrast, other expansion methods, such as those using membrane bound IL-15, reduced telomere length, inducing NK senescence by three weeks.

We believe osteosarcoma can provide an ideal target for NK cell immunotherapy. *In vitro* studies have demonstrated high NK cytotoxicity against pediatric solid tumor cell lines [40]. Among those susceptible to NK killing are Ewing's Sarcoma and osteosarcoma [40-42]. Studies have also report elevated expression of specific NK activating ligands in osteosarcoma [42]. Additionally, NK activity and NK absolute counts correlated positively with clinical outcome in osteosarcoma patients treated with interleukin-2 [43]. Recent studies have also reported the feasibility and safety of using NK cell therapy against solid tumors in the lung [44]. Thirty minutes after transfusion, the lung serves as a reservoir for allogeneic NK cells, before they redistribute to the rest of the body [45]. This makes the lung an ideal organ for NK therapy.

Aerosol Interleukin-2

As mentioned before, most clinical trials using NK adoptive therapy use concomitant interleukin-2 (IL-2) to maintain NK activation *in vivo* [34-36]. Even trials using *ex vivo* NK expansion require IL-2. The highest doses of NK cells to be infused into patients were generated through ex vivo NK expansion using IL-2, OKT3 and PBMC feeder cells. Patients received an average of 4.7×10^{10} NK cells and these cells were persistent in the peripheral circulation. However, tumor regression was not observed, and the persistent NK cells showed significantly lower levels of NKG2D expression and could not lyse tumor cells *in vitro* unless reactivated with IL-2 [37]. This result suggests that even with high NK numbers, systemic IL-2 is required for NK cytotoxicity *in vivo*.

IL-2 is a cytokine that augments the growth and cytotoxicity of T and NK cells, as well as driving T cell differentiation and increasing B cells' antibody production [46]. It is a FDA-approved therapeutic for metastatic melanoma [47] and renal cell carcinoma [48]. However, high dose IL-2 therapy is complicated by serious, sometimes life threatening side effects, including oliguria, hypotension, elevated bilirubin and creatinine levels, thrombocytopenia, and capillary-leak syndrome, which can lead to fluid retention, adult respiratory distress syndrome and myocardial infarction [49].

We propose to use aerosol IL-2 in order to increase the number of injected NK cells numbers in the lung, while at the same time avoiding the systemic toxicities associated with IL-2. The safety of aerosol IL-2 has been established by several clinical trials. Aerosol IL-2 produces only low to mild toxicities [50]. Cough is the most common side-effect [51]. Patients treated with aerosol IL-2 report a better quality of life than those treated with systemic IL-2 [52]. Furthermore, an increased survival has been reported for patients with lung metastasis from renal cell carcinoma treated with aerosol IL-2 [50, 53, 54]. Aerosol IL-2 recruits activated lymphocytes to the lung periphery in a dose-dependent manner, as measured by broncho-alveloar lavage [BAL] [55]. Aerosol IL-2 therapy in dogs has also shown very minimal toxicity. As in humans, BAL revealed an increase in activated lymphocytes [56].

Normally, transfused allogeneic NK cells aggregate in the lung for the first thirty minutes. This is followed by rapid redistribution to the whole body, with a predilection for the liver, spleen and bone marrow [45]. We believe that giving

aerosol IL-2 concomitantly with NK cells will cause the NK cells to be retained and expanded in the lung.

Aim of Study

Our central hypothesis is that aerosol IL-2 will augment the efficiency of NK cell therapy against osteosarcoma lung metastasis by increasing their numbers in the lung, enabling greater contact and interaction with their target cells. We first determined osteosarcoma's expression of NKG2D ligands as well as its susceptibility to NK cytotoxicity. Using *Nu/Nu* mice we also determined if aerosol IL-2 increases the number of injected NK cells in the lung. We proceeded to determine if aerosol IL-2 increases the efficiency of NK cell therapy in mice with osteosarcoma lung metastasis.

RESULTS

Chapter 2

NK cells kill osteosarcoma cells in a NKG2D dependent manner

RATIONALE

The recognition of target cells by NK cells is a complex interplay between inhibitory and activating NK receptors and their respective ligands. The killer-cell immunoglobulin-like receptors [KIRs] are the major inhibitory receptors on human NK cells. They recognize specific HLA-B and HLA-C allotypes [57]. NKG2D is a major NK activating receptor, specifically associated with tumor surveillance [58]. NKG2D recognizes MIC A/B [59] and the ULBP proteins [60], which are overexpressed on several cancers [25, 27, 61]. NK-based immunotherapy is dependent on tumor NKG2D ligand status [58, 62, 63]. In order to determine if NK cell therapy could be an effective tool against osteosarcoma, we determined the NKG2D ligand expression on five different osteosarcoma cell lines. HLA-A,B,C expression was also evaluated. NK cytotoxicity against these cell lines was determined and correlated with NKG2D ligand expression. In addition, to further examine the dependence of NK cytotoxicity on NKG2D ligand expression, the NKG2D –NKG2D ligand interaction was blocked, with monoclonal antibodies.

RESULTS

Human osteosarcoma cell lines express NKG2D ligands

NKG2D ligands are highly expressed among LM7, CCHOSD, U2OS and TE-85 osteosarcoma cells (Figure 1). With the exception of KRIB, all cell lines expressed a high percentage (from 85 to 99%) 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[64], U2OS [65], CCH-OS-D [66] and TE-85 [65]

are non-transformed cell lines derived from patient samples, while KRIB was derived from HOS transfected with v-K-*ras* [65]. This may explain the difference between KRIB cells and the 4 other cell lines.

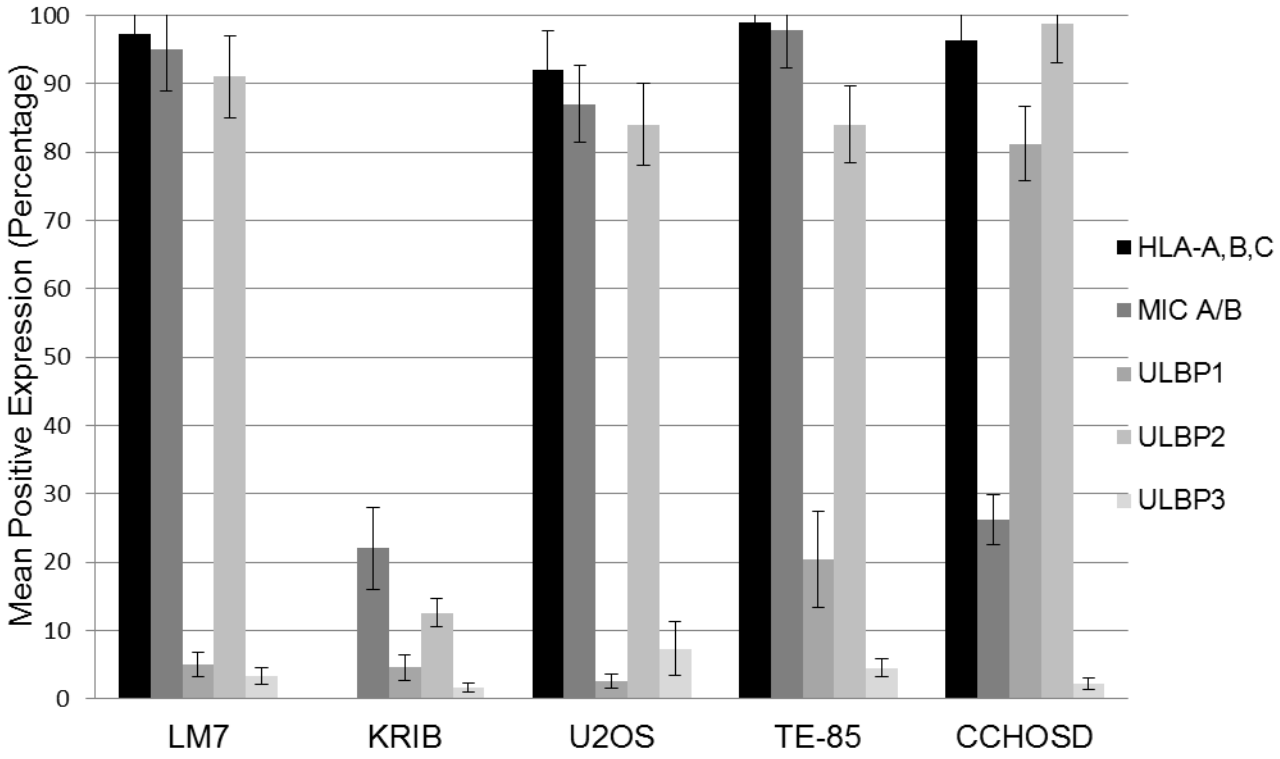


Figure 1: NKG2D Ligand Expression. Human osteosarcoma cell lines were analyzed for the expression of NKG2D ligands and HLA-ABC using flow cytometry**.** LM7, U2OS, TE-85 and CCHOSD cells expressed high levels of NKG2D ligands. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Human NK cells are cytotoxic against human osteosarcoma cells

We determined NK cytotoxicity against LM7, CCH-OS-D, KRIB, TE-85 and U2OS. Since NK-mediated cytotoxicity is species specific, K7M3 mouse osteosarcoma cells were used as the negative control. NK cells were cytotoxic to

all five human cell lines. KRIB cells were the least sensitive to NK-mediated killing (Figure 2). At 1:1 effector: target [E:T] ratio NK cell-mediated cytotoxicity against LM7, CCH-OS-D, U2OS and TE-85 cells was 45% compared to 10% cytotoxicity against KRIB cells. The low level of NKG2D ligands on KRIB cells correlates with the lower cytotoxicity value.

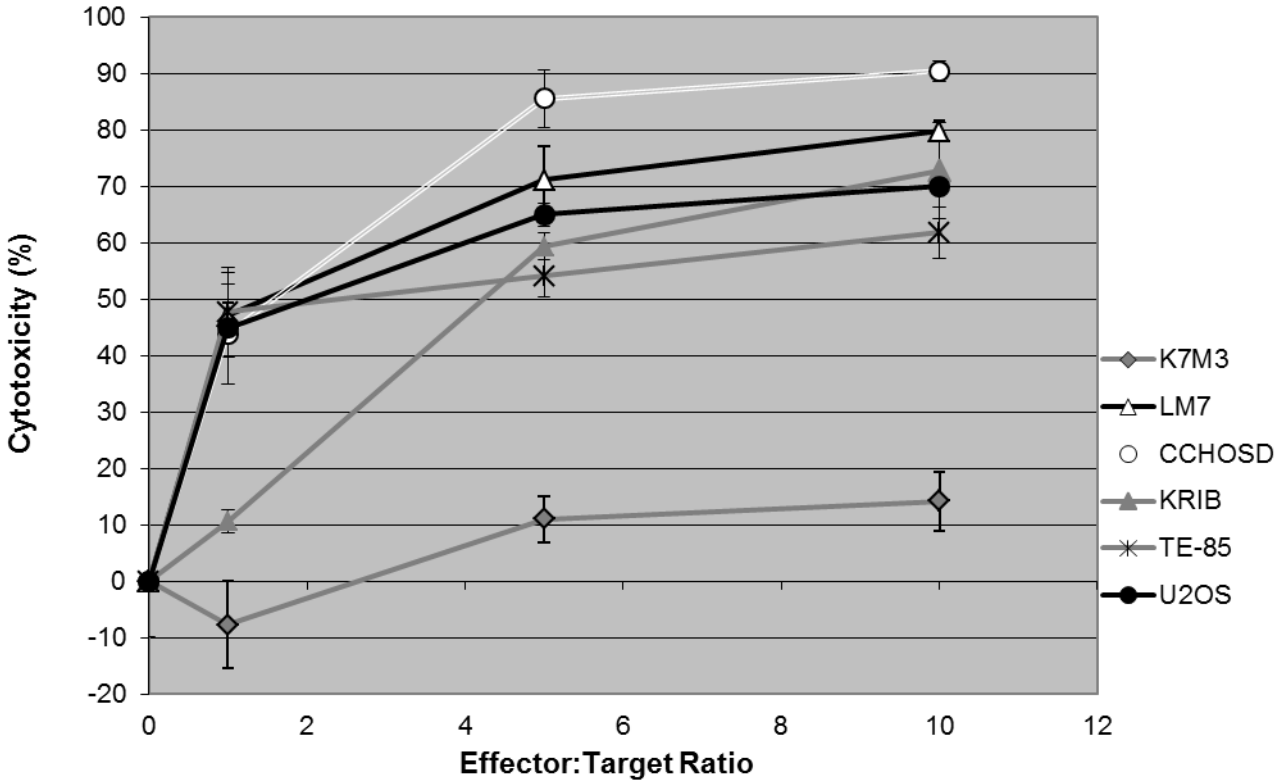
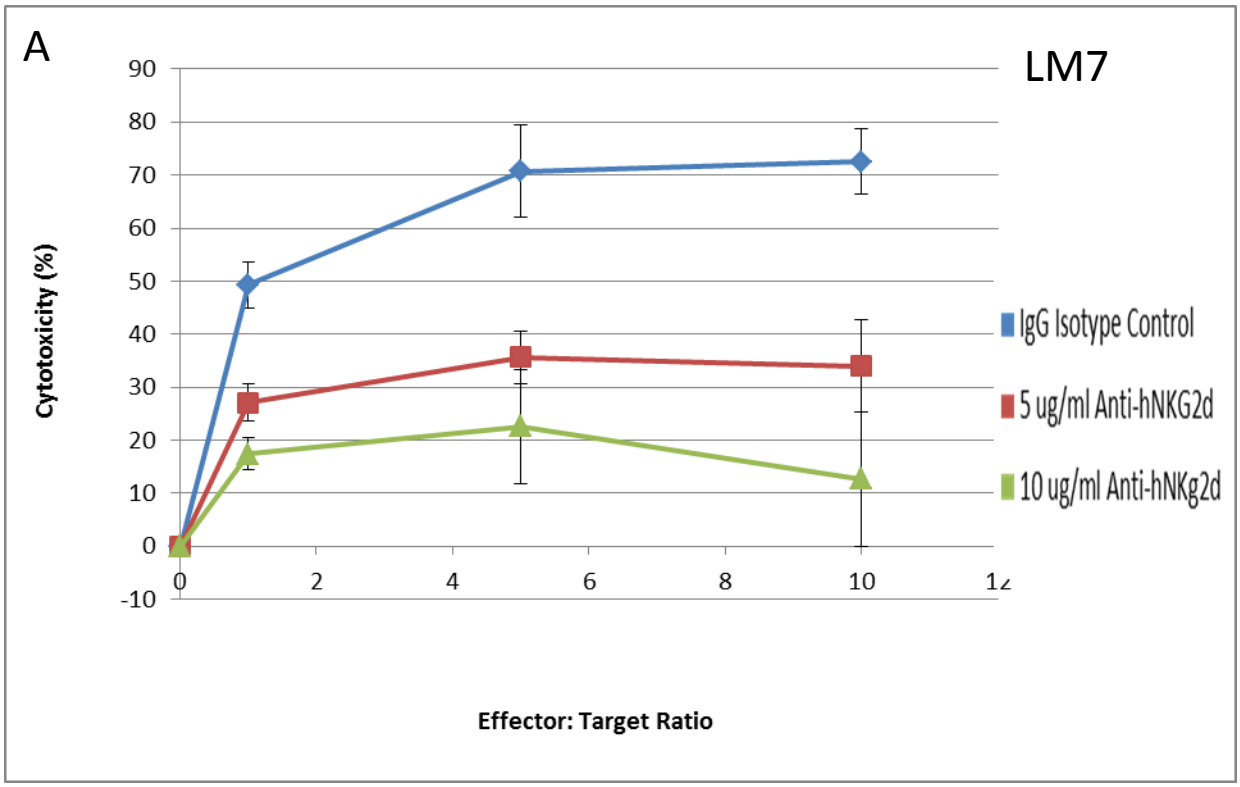


Figure 2: NK-cell mediated cytotoxicity. NK-cell mediated cytotoxicity against KRIB, LM7, TE-85, U20S and CCH-OS-D osteosarcoma cells was quantified using [^{3}H]-thymidine cytotoxicity assay. K7M3 mouse osteosarcoma cells served as the negative control. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

NK cytotoxicity against osteosarcoma is dependent on NKG2D-NKG2D ligand interaction

NK 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 significantly reduced NK cytotoxicity against LM7 cells (Figure

3A). At 1:5 E:T ratio, 10 µg/ml of anti-NKG2D antibody 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). Similarly blocking the ligand also decreased NK-mediated killing (Figure 3B). ULBP2 was selected since this ligand is expressed on all the non-transformed cell lines. Blocking ULPB2 in LM7 significantly reduced NK mediated killing. At 1:5 E:T ratio, 10 µg/ml of anti-ULBP2 antibody decreased cytotoxicity from 70% ± 15 to 29.8% ± 10 (P = 0.004). At a 1:10 E:T ratio, cytotoxicity decreased from 73% ± 6.2 to 39% ± 9.4 (P= 0.01).



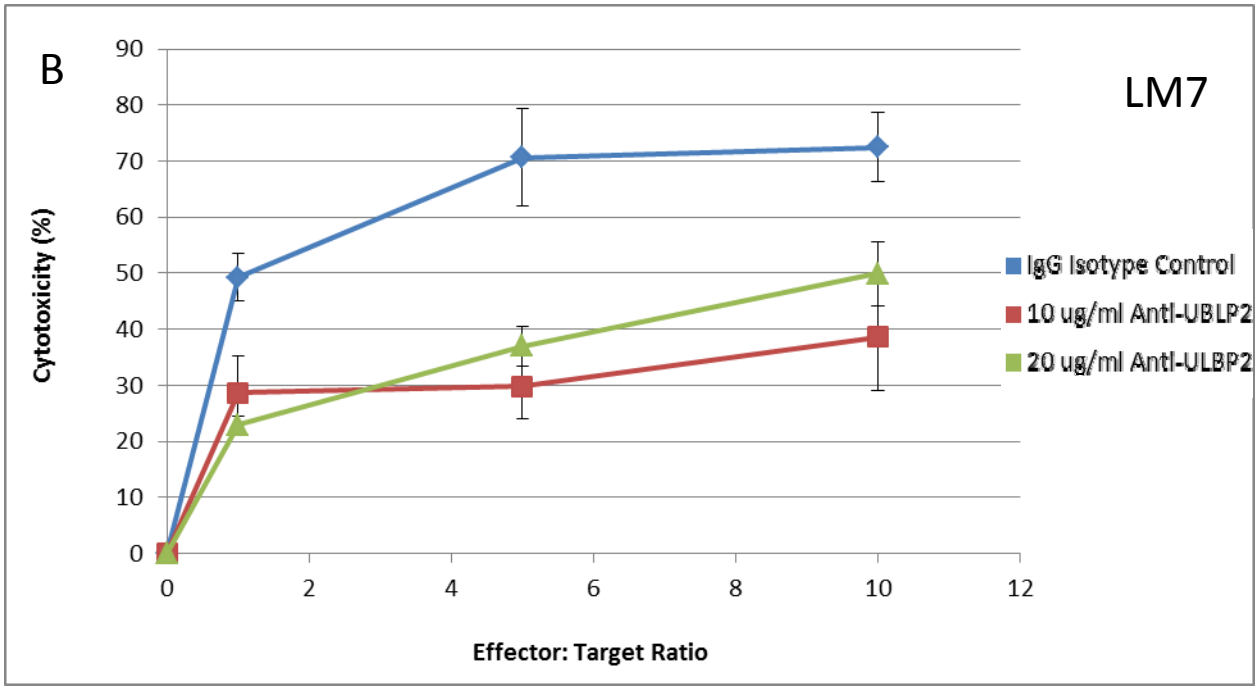


Figure 3: Blocking NKG2D- NKG2D ligand interaction hinders NK cytotoxicity against osteosarcoma A. NK cells were incubated with anti-hNKG2D prior to the addition to LM7 cells. **B.** LM7 cells were incubated with anti-hULBP2 prior to addition of NK cell. $P<0.05$ was considered significant.

SUMMARY

These data support the concept of using NK immunotherapy against osteosarcoma. I have demonstrated that human osteosarcoma cell lines express NKG2D ligands. Accordingly, human NK cells are cytotoxic against these cell lines. The exception was the KRIB cell line, which had very low levels of NKG2D ligand expression. The fact that the KRIB cell line was derived from HOS with a v-K-ras transfection, while the others are non-transformed cell lines derived from patient samples, may explain KRIB's low NKG2D ligand expression and,

consequently, lower NK sensitivity. I further demonstrated that by blocking the NKG2D-NKG2D ligand interaction, NK cytotoxicity was hindered. These results support our hypothesis that NK cytotoxicity against osteosarcoma correlates and is dependent on NKG2D ligand expression. In potential future clinical trials, the effectiveness of NK cell therapy against osteosarcoma may be predicted by the levels of NKG2D ligand expression.

RESULTS

Chapter 3

The expression of NKG2D ligands in osteosarcoma patient samples.

RATIONALE

After demonstrating that human osteosarcoma cell lines have an increased NKG2D ligand expression as well as a high sensitivity to NK killing, we further investigated the plausibility of NK cell therapy by determining the expression of NKG2D ligands in patient osteosarcoma samples. Previous investigations [58, 62, 63] have demonstrated that the status of NKG2D ligand expression is important for immune surveillance and for effective cytokine therapies. We have confirmed that NK cytotoxicity against osteosarcoma correlates with and is dependent on NKG2D-NKG2D ligand interaction. Therefore, we can predict the effectiveness of NK cell based immunotherapy by determining the level of osteosarcoma NKG2D ligand expression.

Paraffin-embedded microarray slides of patient osteosarcoma tissues from the University of Michigan were kindly provided by Dr. Dennis Hughes, MD/PhD (Division of Pediatrics, The University of Texas MD Anderson Cancer Center). The tissue microarray slides contained 47 primary osteosarcoma and 56 osteosarcoma lung metastasis samples. We determined NKG2D ligand expression through immunohistochemistry using a recombinant human NKG2D/Fc Chimera.

RESULT

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%) (Figure 4A). 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%) (Figure 4B). (*Originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc.)*

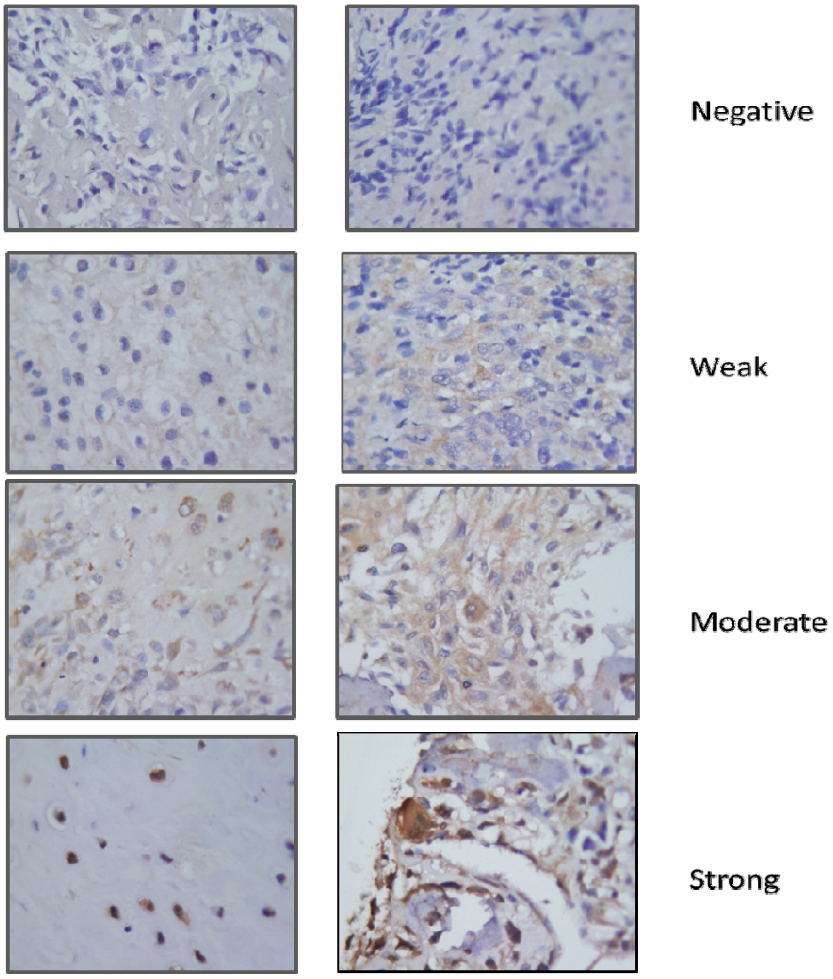


Figure 4: Expression of NKG2D ligands on human osteosarcoma tumors. Human osteosarcoma patient samples from primary tumor (**A**) and lung metastasis (**B**) were analyzed for the expression of NKG2D ligands by immunohistochemistry using a rhNKG2D/Fc. Positive NKG2D ligand staining was classified as negative, weak, moderate or strong. *Figure and figure legend were originally published as supplemental material in Pediatric Blood and Cancer 2013). This material is reproduced with permission of John Wiley & Sons, Inc..*

SUMMARY

The data also demonstrate that a high percentage of osteosarcoma express NKG2D ligands. It also demonstrates that osteosarcoma pulmonary metastasis has a higher NKG2D ligand expression than primary tumors. This results validate that osteosarcoma lung metastasis could be an ideal target for NK cell therapy.

RESULTS

Chapter 4

Aerosol IL-2 increases the number and proliferation of NK cells in the lung

RATIONALE

In the previous chapters, we have shown that osteosarcoma cells are sensitive to NK cell killing and that both osteosarcoma cell lines and patient tissue samples expresses the ligands required for this sensitivity. However, for NK cell therapy to be effective, a sufficient number of NK cells must come into contact with their target cells. Since we are specifically interested in pulmonary osteosarcoma metastasis, we proposed using aerosol IL-2 to increase the number of injected NK cells present in the lung.

IL-2 is a cytokine that stimulates the growth, cytotoxicity and survival of NK cells [67]. For this reason, it has been given as an *in vivo* activator in clinical studies involving NK cell therapy [68, 69]. Yet, high dose IL-2 induces a myriad of life-threatening side effects, including oliguria, hypotension, and elevated bilirubin and creatinine levels [68]. Furthermore, its effects are not organ specific. We proposed using aerosol IL-2 to stimulate the injected NK cells in the lung specifically, the most frequent site for osteosarcoma metastasis [70].

Nu/Nu mice were injected with human NK cells and treated with either aerosol IL-2 or aerosol PBS. The effect of aerosol IL-2 on NK cell numbers in the lung, liver, spleen, heart and kidney was determined. To further corroborate these findings, we determined the percentages of proliferating NK cells in the lungs, spleen, liver and bone marrow.

RESULTS

Aerosol IL-2 increased NK cells in the lungs

Aerosol IL-2 increased the number of NK cells in the lung 3 days after human NK cell injection ($P = 0.02$; Figure 5). There was a slight increase 1 day after injection when compared to control yet not in a statistically significant manner ($P = 0.26$). The number of NK cells in the lung for aerosol PBS treated mice did not change between 1 day, 3 days and 1 week after NK cell injection. After one week, the number of NK cells in the lung of aerosol IL-2 treated mice decreased to levels similar to aerosol PBS-treated mice, suggesting aerosol IL-2's effect on the injected NK cells' pulmonary presence is time-dependent.

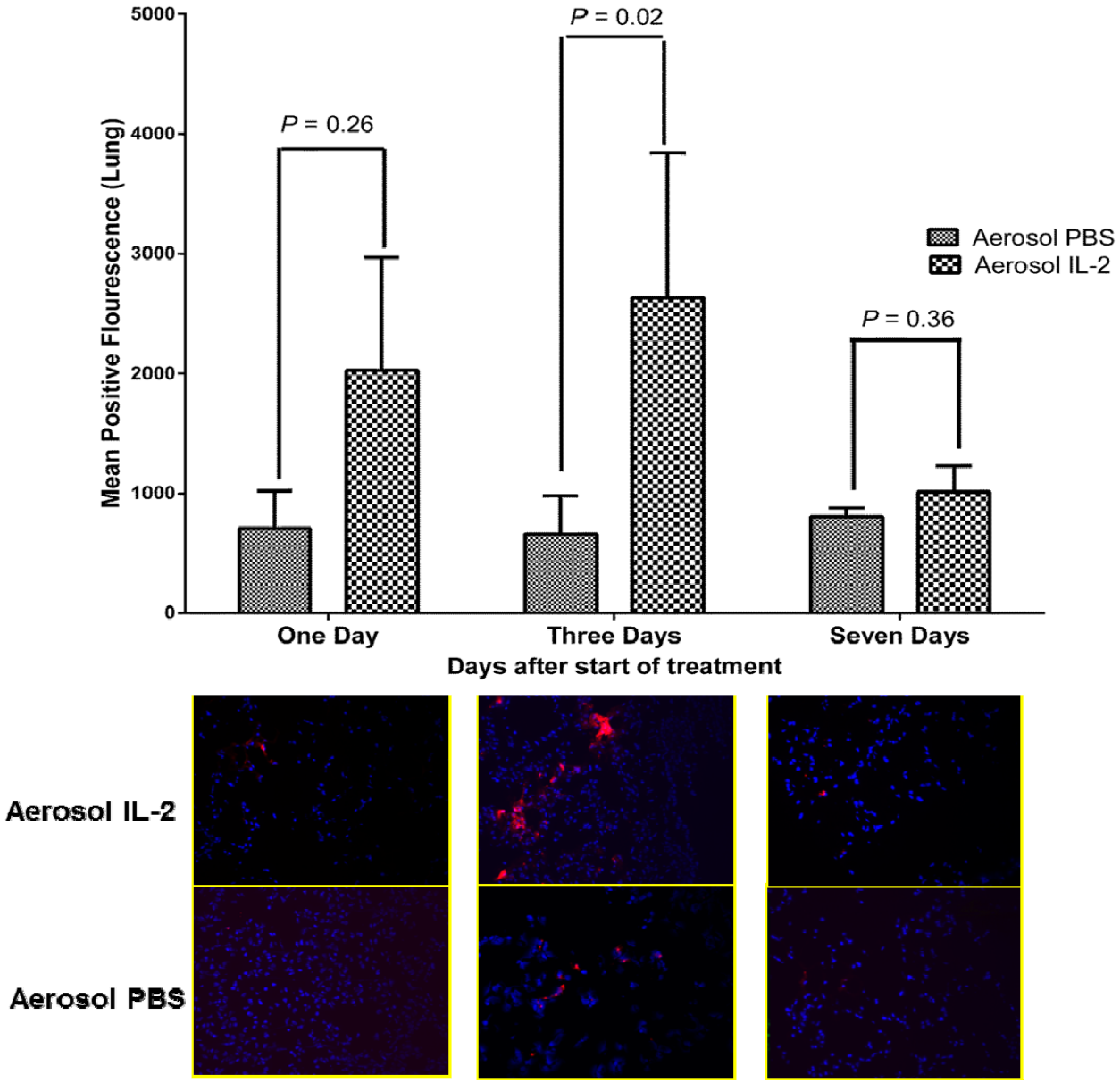


Figure 5. Aerosol IL-2 increases human NK cells in the lungs. Nude mice were injected with $5x10^7$ CM-DiI-labeled human NK cells per mouse. Aerosol PBS or IL-2 was given 1 day prior to and on the day of NK cell infusion and then continued every other day for 1 week. Fluorescent microscopy was used to detect the presence of CM-DiI+ 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. Representative pictures of NK cell presence in the lung at the different time points are shown. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Aerosol IL-2 did not increase NK cells in the spleen, liver, heart or kidney

High numbers of injected NK cells were observed in the spleen and liver (Figure 6 A,B), suggesting that a large portion of infused NK cells localize into these two organs. However, there was no aerosol IL-2-associated increase. It is worthwhile to note that there is a non-statistically significant decreasing trend in the number of NK cells present in these organs when aerosol IL-2 was given. This is consistent with the increased presence of NK cells seen in the lung. There was a very minimal presence of injected NK cells in the heart and kidney (Figure 6 C,D) with no increase associated with aerosol IL-2 treatment.

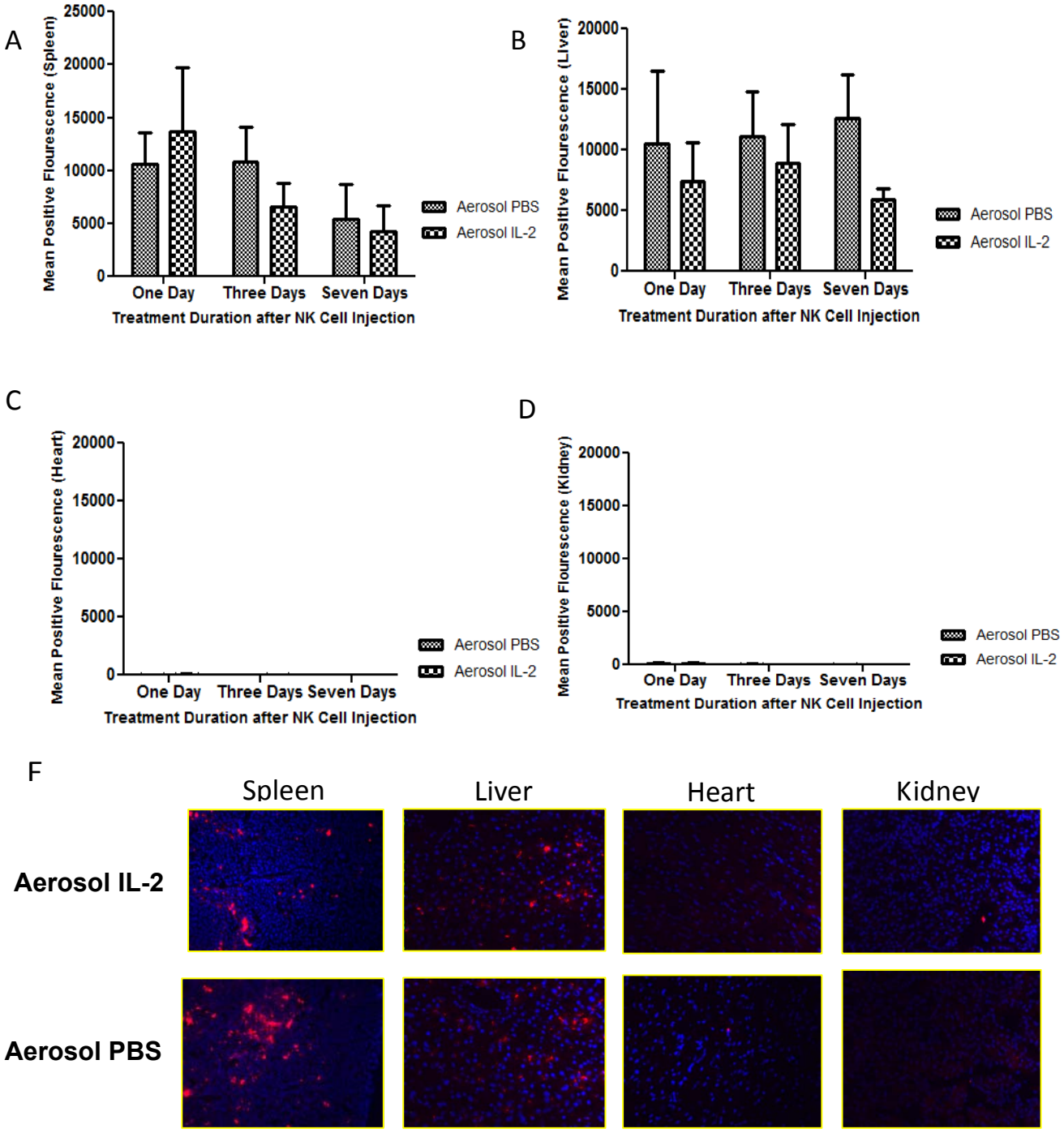


Figure 6. Aerosol IL-2 does not increase human NK cells in the spleen, liver, heart or kidney. Mean positive fluorescence was quantified for the presence of CM-DiI+ NK cells in **A** spleen, **B** liver, **C** heart and **D** kidney. **E**. Representative sections from organs 3 days after NK cell infusion. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Aerosol IL-2 increased the proliferation of NK cells in the lung

We proceeded to determine if the increase in lung NK cells caused by aerosol IL-2 was due to an increase in NK cell proliferation. For example, the increase of mouse NK cells observed in the lung after intranasal respiratory viral infection is not due to an increase of NK cell proliferation in the lung. Instead it is caused by an increase in proliferation of the NK cells in the bone marrow [71]. To determine if that is the case for aerosol IL-2 therapy, we injected *Nu/Nu* mice with human NK cells and treated them with either aerosol PBS or aerosol IL-2. We injected these mice with BrdU 3 hours prior to euthanasia and harvested cells from their lungs, spleen, liver and bone marrow. Cells that stained positive for human NKp46 and BrdU were identified as proliferating NK cells; cells that were $NKp46^{+}/BdU^{-}$ were identified as non-proliferating NK cells.

Aerosol IL-2 increased human NK proliferation in the lung. This was seen by a marked increase in the percentage of proliferating NK cells seen 24 and 72 hours after NK injection (Figure 7). 24 hours after injection, 20% ± 4 of the human NK cells in the lung of mice treated with aerosol IL-2 were proliferating, compared to 8% ± 0.15 of those in the lung of aerosol PBS treated mice (P = 0.03). The difference between treatments increased over time. 72 hours after injection, 76% ± 9.5 of the human NK cells in the lung of mice treated with aerosol IL-2 were proliferating, compared to 9.2% ± 6.2 of those in the lung of mice treated with aerosol PBS (P = 0.007).

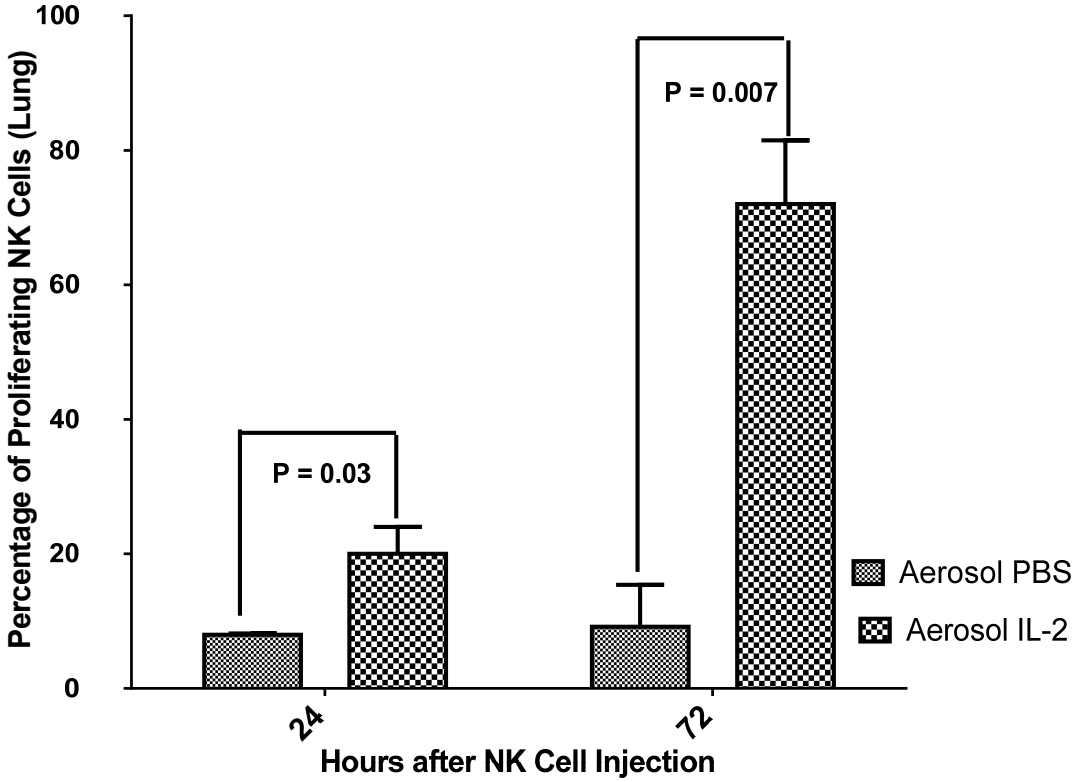


Figure 7: Aerosol IL-2 increases the percentage of proliferating NK cells in the lung. Nude mice were injected with $5x10^7$ human NK cells per mouse. Aerosol PBS or IL-2 was given 1 day prior to and on the day of NK cell infusion and then continued every other day. BrdU reagent was injected intraperitoneally 3 hours prior to euthanasia. Lungs were harvested and minced. Single-cell suspensions were prepared by passage through cell strainers. Flow cytometry was used to identify NK cells with anti-human NKp46 staining and proliferating cells with anti-BrdU staining. $P< 0.5$ was considered significant.

Aerosol IL-2 increased the proliferation of NK cells in the bone marrow

A large percentage of human NK cells were proliferating in the bone marrow, in both aerosol IL-2 and aerosol PBS treated mice (Figure 8). However, there was an increase in the percentage of proliferating NK cells found in the bone marrow of mice treated with aerosol IL-2. 24 hours after injection, 93.6 % ± 4.8 of the human NK cells in the bone marrow of mice treated with aerosol IL-2

were proliferating, compared to 65.5% ± 0.15 of those in the bone marrow of aerosol PBS treated mice (P = 0.03). 72 hours after injection, the percentage of proliferating NK cells in the bone marrow decreased. 58.5 % ± 1.5 of the human NK cells in the bone marrow of mice treated with aerosol IL-2 were proliferating, compared to 23.8% ± 8.9 of those in the bone marrow of mice treated with aerosol PBS. At 72 hours after injection, the difference between the percentage of proliferating NK cells between the two treatment groups was not statistically significant (P = 0.058).

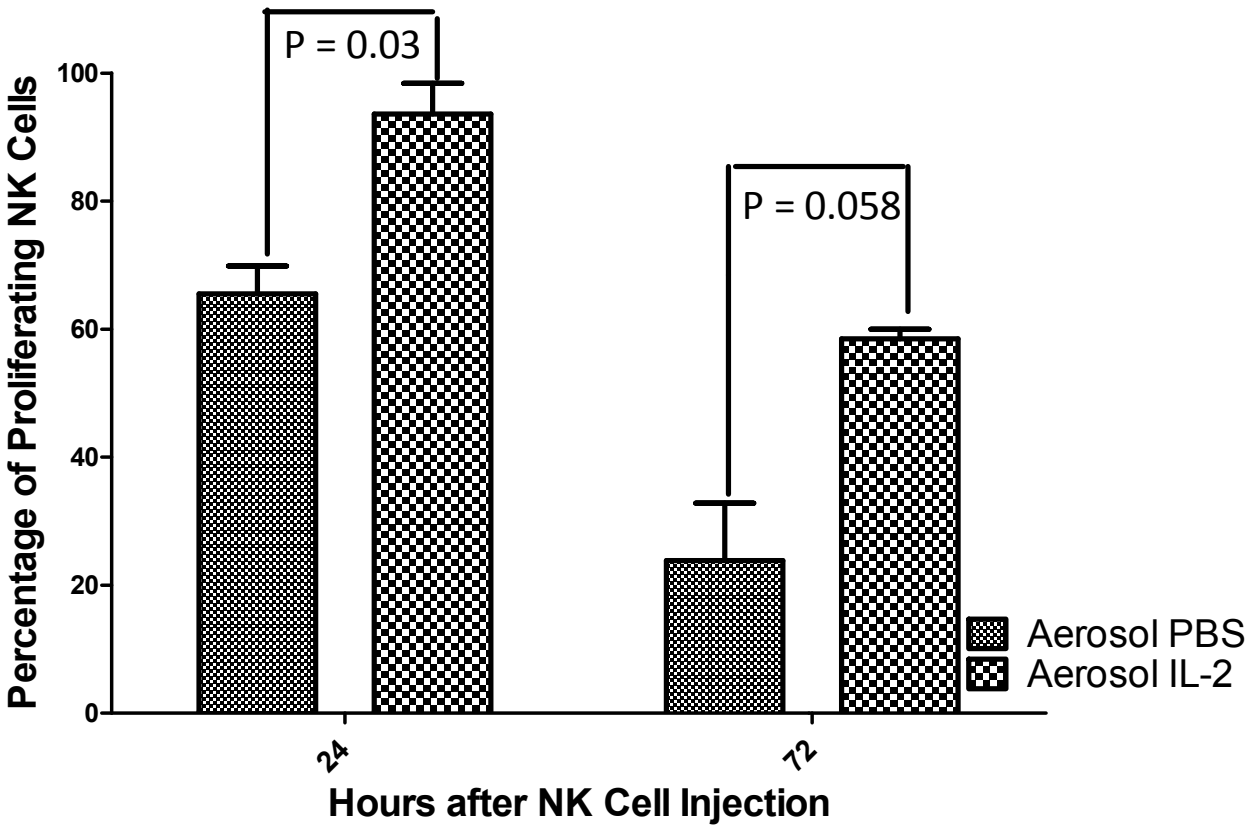


Figure 8: Aerosol IL-2 increases the percentage of proliferating NK cells in the bone marrow. Nude mice were injected with $5x10^7$ human NK cells per mouse. Aerosol PBS or IL-2 was given 1 day prior to and on the day of NK cell infusion and then continued every other day. BrdU reagent was injected intraperitoneally 3 hours prior to euthanasia. Bone marrow cells were obtained by flushing femurs and iliac crest with PBS. 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.

Aerosol IL-2 did not increase the proliferation of NK cells in the spleen or liver

In both the aerosol IL-2 and aerosol PBS treated-mice, a very low percentage of NK cells were proliferating in the spleen and liver 24 hours after NK cell injection (Figure 9). In the spleen, 2.55 % ± 2.55 of the human NK cells of mice treated with aerosol IL-2 were proliferating, compared to 2.3% ± 2.23 of

human NK cells in mice treated with aerosol PBS (P = 0.9). In the liver, 5.1 % ± 1.1 of the human NK cells of mice treated with aerosol IL-2 were proliferating, compared to 4.2% ± 1.6 of human NK cells in mice treated with aerosol PBS (P = 0.72). The difference between the percentages of proliferating NK cells between the two treatment groups was not statistically significant. At 72 hours, the percentages of proliferating NK cells increased in the two organs, yet the differences between the two groups remained statistically insignificant. In the spleen, 44.1 % ± 8.4 of the human NK cells of mice treated with aerosol IL-2 were proliferating, compared to 23.3% ± 9.1 of human NK cells in mice treated with aerosol PBS (P = 0.1). In the liver, 11.7 % ± 3.8 of the human NK cells of mice treated with aerosol IL-2 were proliferating, compared to 16.3% ± 10.7 of of the human NK cells of mice treated with aerosol PBS (P = 0.74).

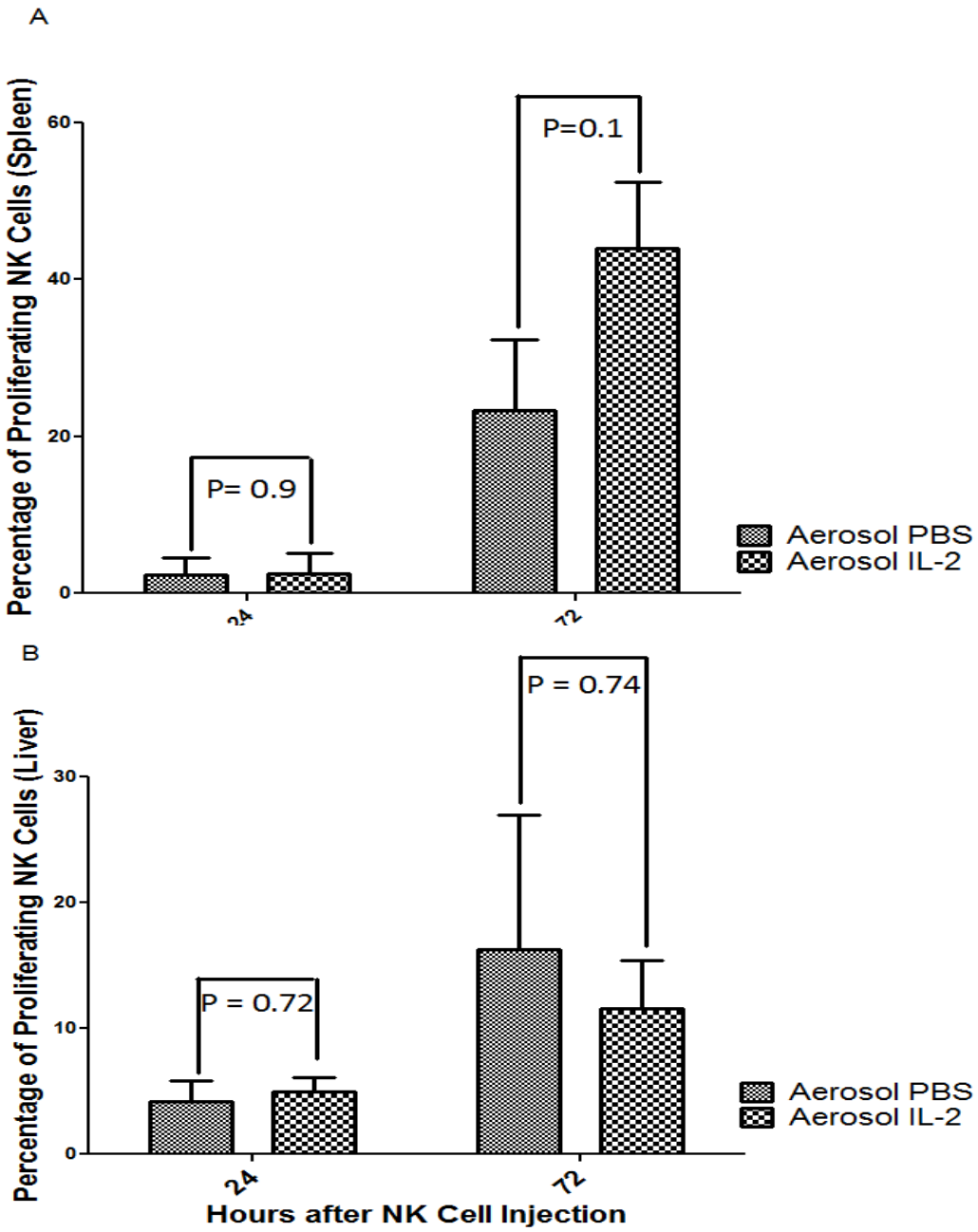


Figure 9: Aerosol IL-2 does not increase the percentage of proliferating NK cells in the spleen or spleen. Nude mice were injected with $5x10^7$ human NK cells per mouse. Aerosol PBS or IL-2 was given 1 day prior to and on the day of NK cell infusion and then continued every other day. BrdU reagent was injected intraperitoneally 3 hours prior to euthanasia. Spleens and livers were harvested and minced. Single-cell suspensions were prepared by passage through cell strainers. 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.

SUMMARY

We have demonstrated that aerosol IL-2 treatment increases the number of injected NK cells in the lung. In contrast, it does not increase the number of NK cells in the spleen, liver, heart or kidney. This result suggests that the effect of aerosol IL-2 on NK cells is organ specific. We further demonstrated that a higher percentage of NK cells in the lung are proliferating when aerosol IL-2 is given. This suggests that aerosol IL-2 is directly stimulating the proliferation of NK cells in the lung. This effect is seen 24 hours after NK cell injection, and continues to increase at least up to 72 hours after NK cell injection.

Aerosol IL-2 did not increase the proliferation of NK cells in the liver or spleen. Aerosol IL-2 slightly increased the proliferation of human NK cells in the bone marrow.

RESULTS

Chapter 5

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

RATIONALE

Our results have indicated that human NK cells are cytotoxic against human osteosarcoma cells *in vitro*. Our results have also indicated that aerosol IL-2 increases the number of injected NK cells in the lung. We hypothesized that in the presence of pulmonary osteosarcoma metastasis, aerosol IL-2 would increase the number of NK cells that come in contact with their target tumor cells, enhancing killing. In this chapter, I describe how we tested this hypothesis.

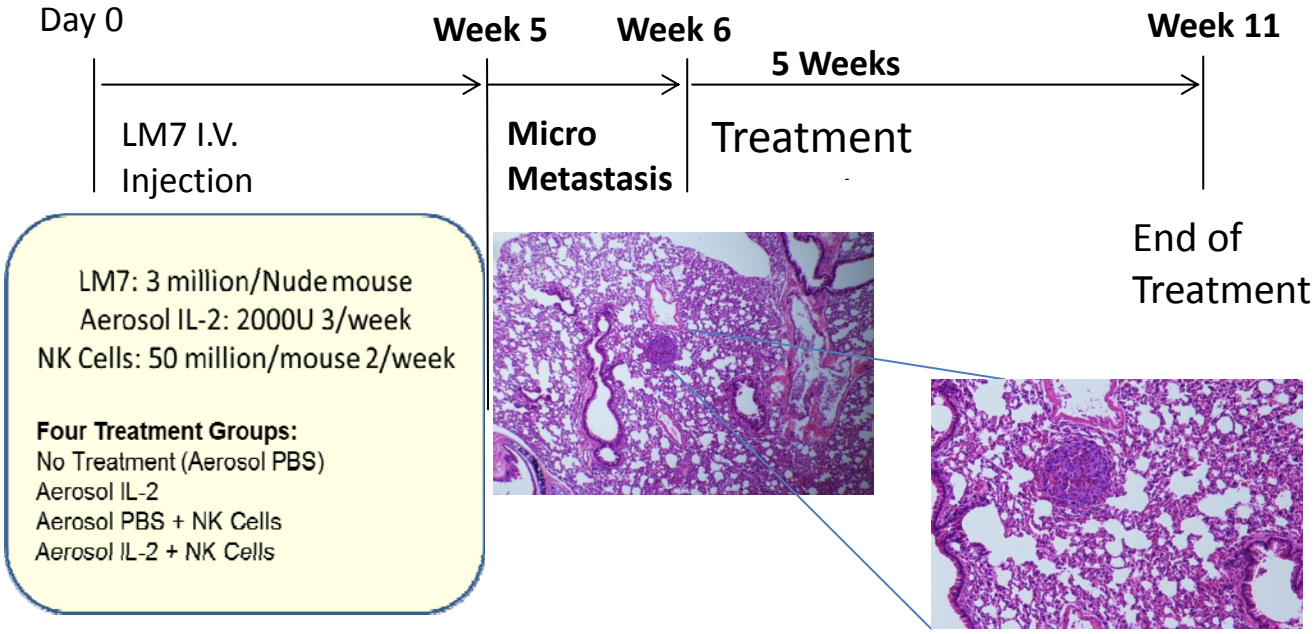


Figure 10. Schematic diagram of in vivo model experimental design. Nude mice were injected i.v. with 3 x 10^6 LM7 cells. Presence of micrometastasis was confirmed by H&E at week 5. Therapy was initiated on week 6. Mice were treated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells two times per week for 5 weeks

We used our established osteosarcoma pulmonary metastasis model [64]. 3 million LM7 cells/mouse were injected intravenously through the tail vein of *nu/nu* mice. The presence of micrometastasis was confirmed through H&E at 5 weeks in a group of 3 mice. Treatment was then initiated with aerosol PBS,

aerosol IL-2, aerosol PBS + human NK cells or aerosol IL-2 + human NK cells 6 weeks after LM7 injection. Aerosol therapy was continued every other day for 5 weeks. NK cell injections (5 x 10^7 cells/mouse) were given 2 times a week starting 1 day after the first aerosol treatment. Following the treatment, the mice were sacrificed and the lungs resected for further analysis (Figure 10).

RESULTS

Aerosol IL-2 + NK cell therapy decreases the number of pulmonary metastatic nodules

Aerosol IL-2 + NK cell therapy significantly decreased the number of pulmonary metastatic nodules, when compared to control (aerosol PBS) (P = 0.01), aerosol IL-2 alone (P = 0.03) and aerosol PBS + NK cells (P = 0.01) (Figure11). Four mice treated with aerosol IL-2 + NK had no visible metastasis present in the lung. In comparison, only one of the mice treated with aerosol PBS + NK cells had no visible pulmonary metastasis (Figure 12). The mean number of pulmonary metastatic nodules present in mice treated with both aerosol IL-2 and NK cells was 2.8 ± 1.6, compared to 13 ± 3 nodules for aerosol PBS + NK cell treated mice, 18.4 ± 5.4 nodules for aerosol IL-2 treated mice and 22.6 ± 6 nodules present in aerosol PBS treated mice. Both aerosol IL-2 alone and aerosol PBS + NK cell treatments decreased the mean number of metastatic lung nodules, but the decrease was not statistically significant when compared to aerosol PBS treatment (P = 0.6 and P = 0.1, respectively).

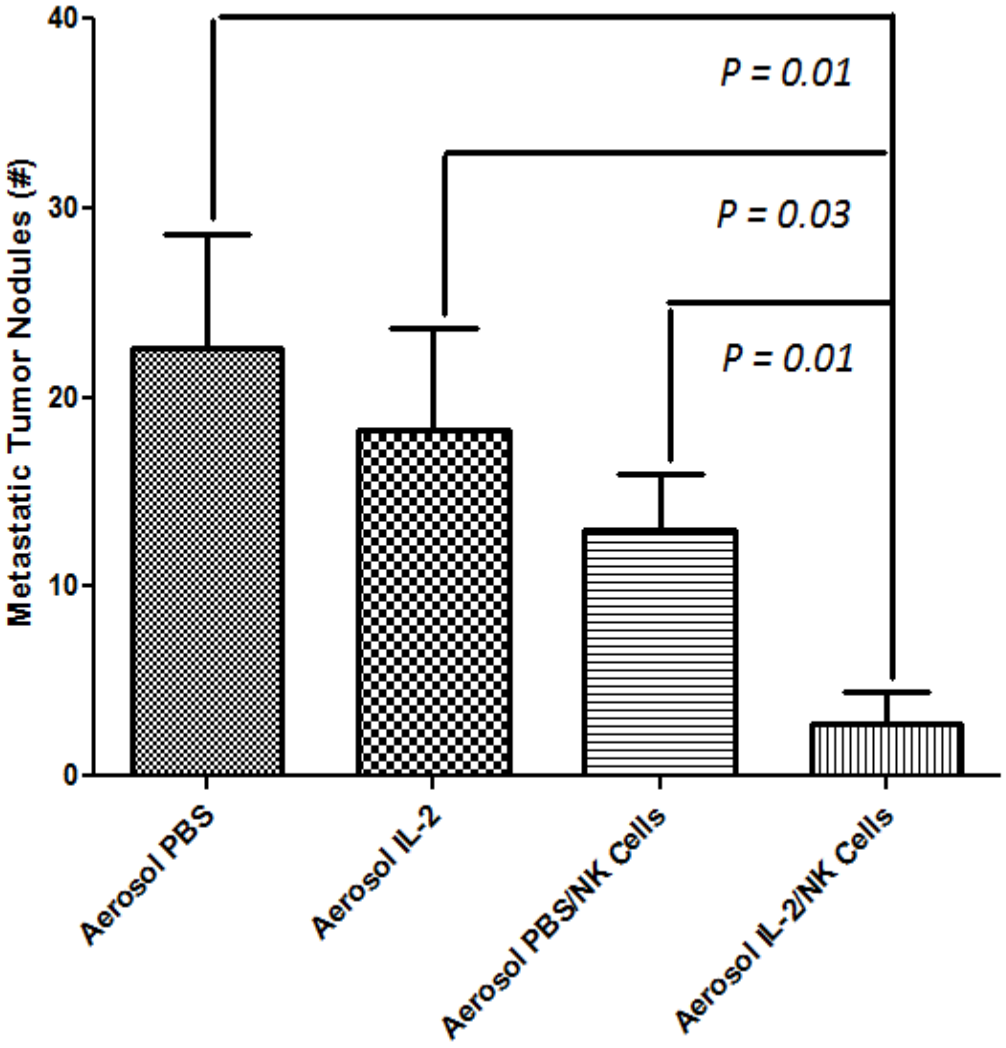


Figure 11: Aerosol IL-2 + NK cell therapy decreases the number of pulmonary metastatic nodules. Nude mice were injected i.v. with 3 X 10^6 LM7 cells. Therapy was initiated 6 weeks later. Mice were treated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells for five weeks. Mice were sacrificed, their lungs harvested and the mean number of metastatic nodules was determined. $P < 0.05$ was considered significant. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

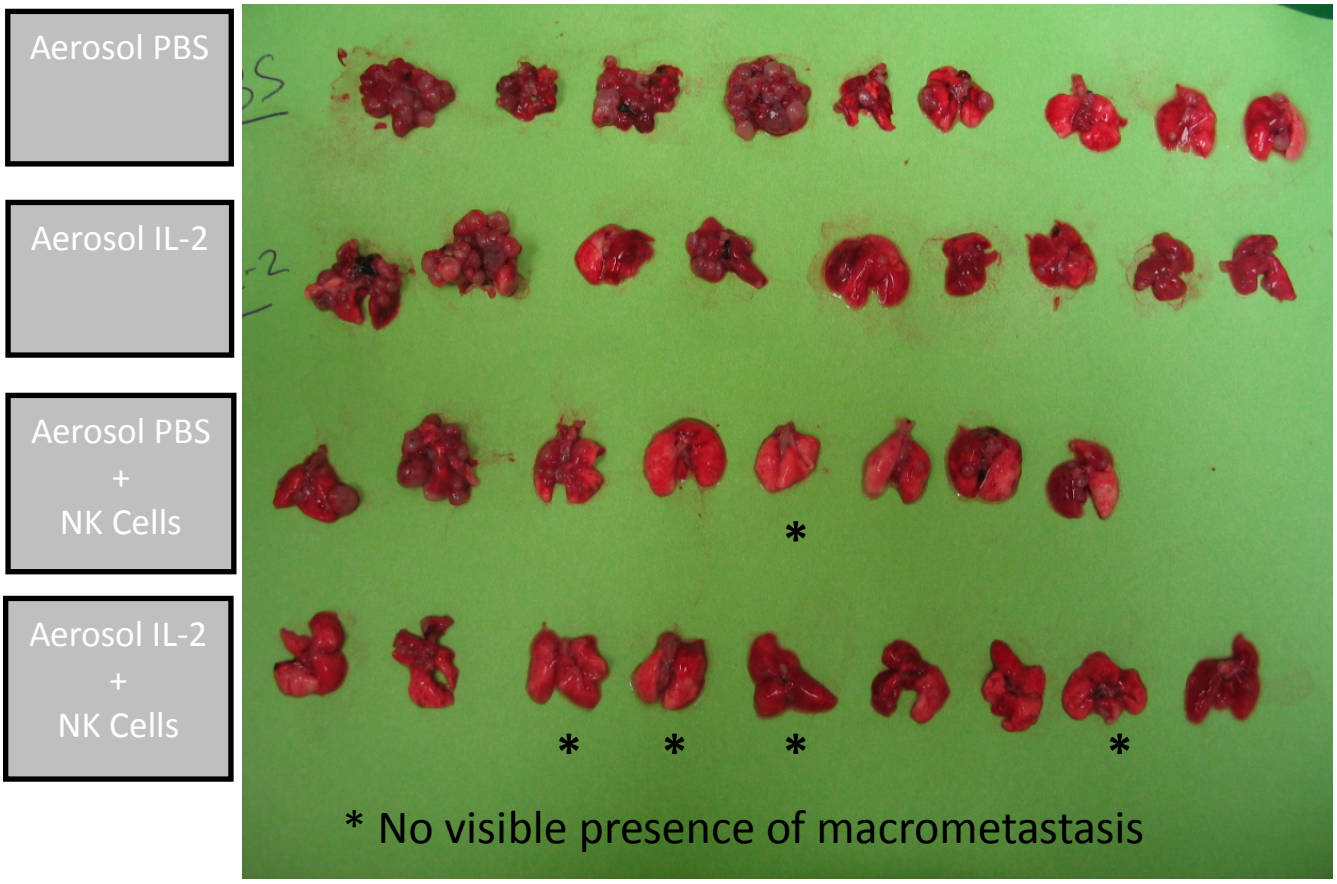


Figure 12: Representative picture of lungs of mice treated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells and aerosol IL-2 + NK cells for five weeks. Nude mice were injected i.v. with $3X10^6$ LM7 cells. Therapy was initiated 6 weeks later. Mice were sacrificed and their lungs harvested. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Aerosol IL-2 + NK cell therapy decreases the size of pulmonary metastatic nodules

Aerosol IL-2 + NK cell therapy significantly decreased the size of the individual lung tumor nodules (Figure 13). The mean diameter of lung nodules in mice treated with aerosol IL-2 + NK cells was 1.12 mm ± 0.36, compared to 2.07 mm ± 0.46 for those treated with aerosol PBS ($P = 0.005$) and 1.9 mm ± 0.42 for those treated with aerosol IL-2 ($P = 0.004$). Aerosol IL-2 therapy alone did not

reduce the mean tumor diameter when compared to aerosol PBS (*P* = 0.8). Aerosol PBS + NK cell therapy did seem to decrease tumor size, from a mean tumor diameter of 2.07 mm ± 0.46 for those treated with aerosol PBS to 1.37 mm ± 0.43. Yet, this decreases was not statistically significant (*P* = 0.07). The mean tumor diameter was not statistically significant between aerosol IL-2 + NK cell and aerosol PBS + NK treated mice (*P* = 0.4).

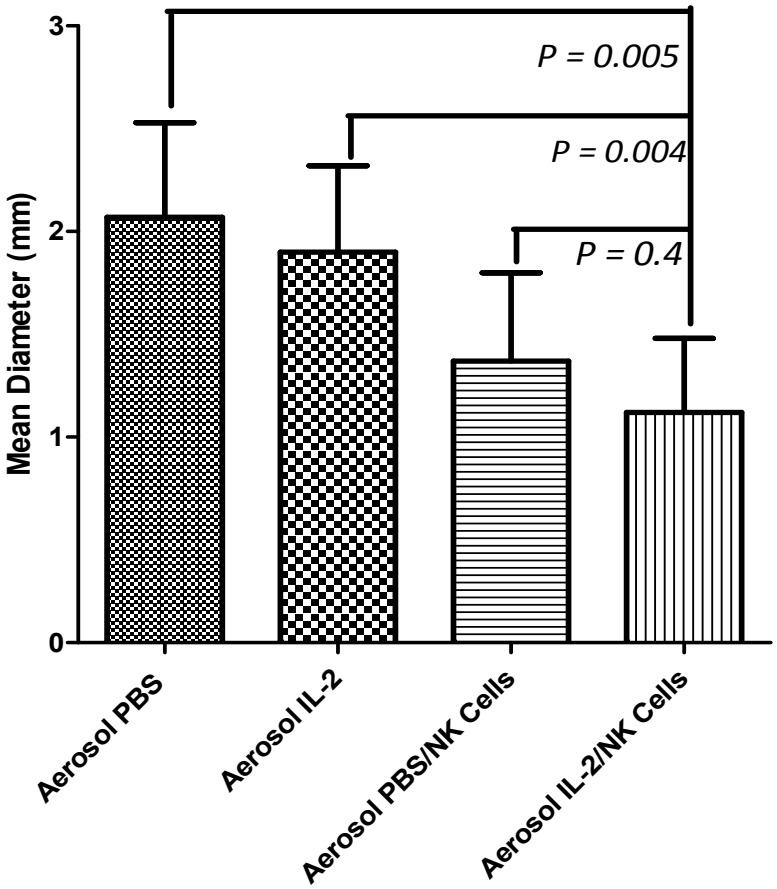


Figure 13: Aerosol IL-2 + NK cell therapy reduces pulmonary metastatic nodule size. Nude mice were injected i.v. with $3X10^6$ LM7 cells. Mice were treated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells for five weeks. Mice were sacrificed, their lungs harvested and the diameter of each individual tumor nodule was measured. *P* < 0.05 was considered significant. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Aerosol IL-2 + NK Cell therapy decreases the total surface area of the lung that was infiltrated with osteosarcoma metastasis

The surface area of each tumor nodule was calculated. The total surface area of each lung that was infiltrated with metastasis was calculated by the sum of the surface areas of each of its individual tumor nodules. From these values, the mean total metastatic area was calculated for each treatment group. Aerosol IL-2 + NK cell therapy significantly reduced the total area of the lungs covered in metastases compared to that of mice treated with aerosol PBS (P =0.004), aerosol IL-2 (P = 0.04) or aerosol PBS + NK cells (P= 0.05) (Figure 14).

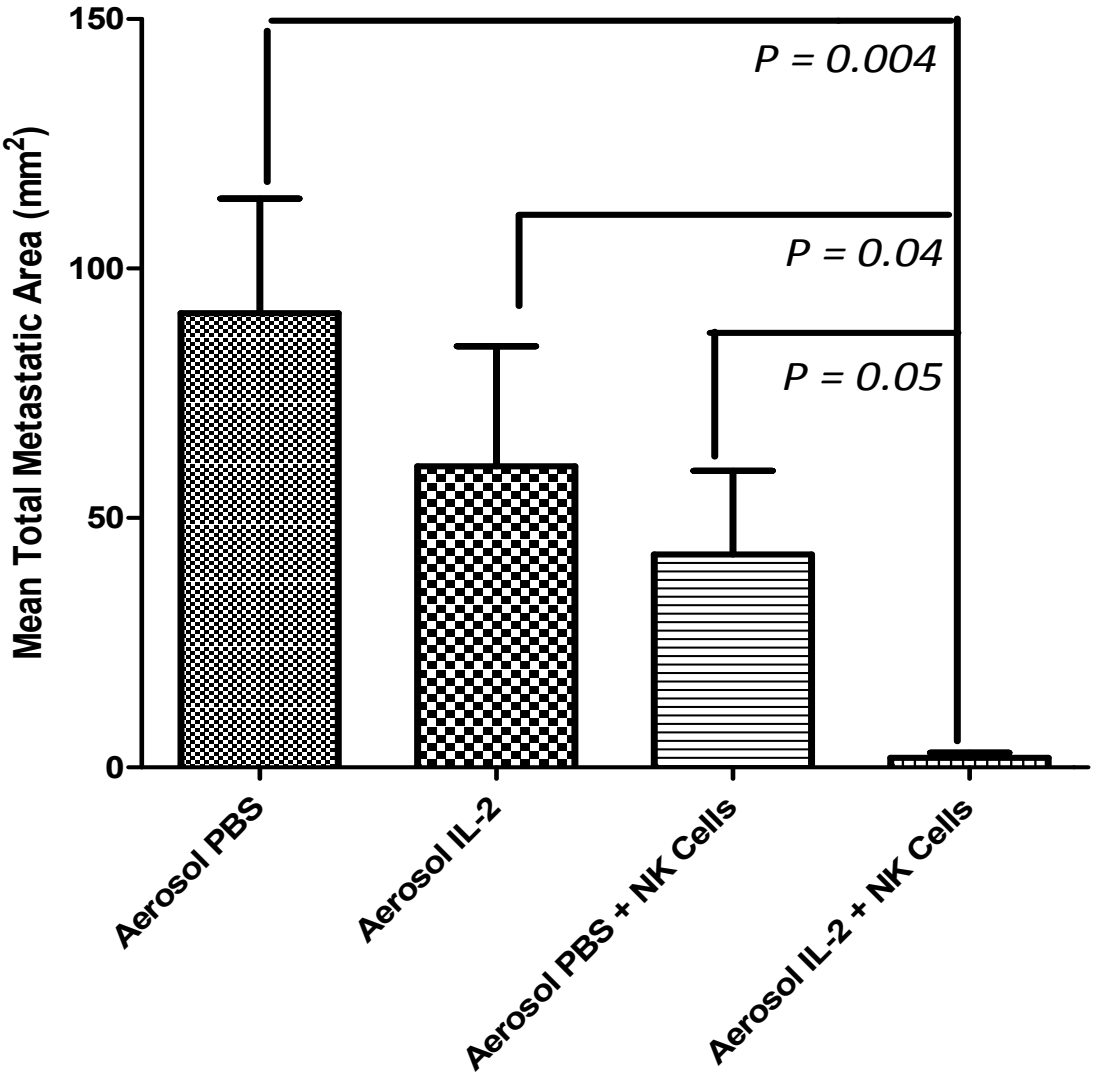


Figure 14: Aerosol IL-2 + NK cell therapy decreases the total area of lungs infiltrated with osteosarcoma metastasis. Nude mice were injected i.v. with 3 X10^6 LM7 cells. Mice were treated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells for five weeks. Mice were sacrificed, their lungs harvested and the total metastatic area was calculated. $P < 0.05$ was considered significant. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Aerosol IL-2 + NK Cell therapy increased tumor apoptosis

NK cell killing is mediated by the secretion of cytotoxic granules that trigger the apoptotic pathway [28]. We have demonstrated that aerosol IL-2 + NK cell therapy significantly reduced metastatic tumor burden. TUNEL staining was used to evaluate if this were due to increased apoptosis in the tumor nodules (Figure 15). Apoptosis was significantly higher in lung metastases from the mice treated with aerosol IL-2 + NK cells when compared to mice treated with aerosol PBS (P =0.009), aerosol IL-2 (P= 0.02) or aerosol PBS + NK cells (P= 0.05). Aerosol IL-2 and PBS + NK cells also increased tumor apoptosis when compared to aerosol PBS treatment. However, though the mean of aerosol IL-2-induced apoptosis was lower than that induced by aerosol PBS + NK cell therapy, its increase was statistically significant when compared to aerosol PBS (P = 0.003), while the increase induced by aerosol PBS + NK cells was not statistically significant (P = 0.17).

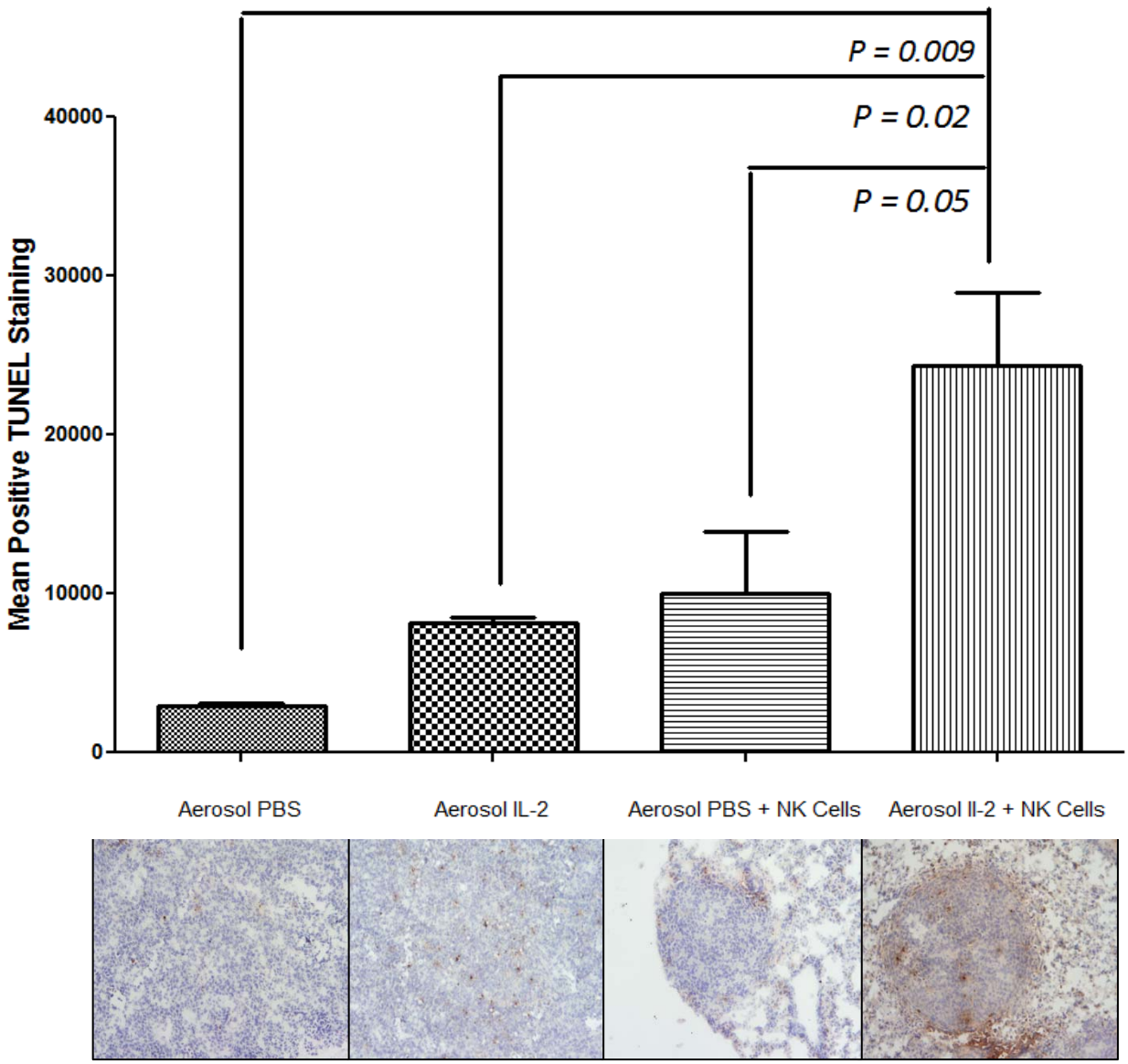


Figure 15. Aerosol IL-2 + NK cell therapy increased tumor apoptosis. Mean positive TUNEL was quantified using the Simple PCI software in 5 random fields per section. $P < 0.05$ was considered significant. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Tumor apoptosis inversely correlates with the amount and size of pulmonary metastatic nodules.

To further corroborate that the increase in apoptosis is associated with the decrease in pulmonary metastatic burden, TUNEL values for each lung were

correlated to the corresponding number of metastatic nodules, mean diameter of nodules, and total metastatic area using a Spearman Rank correlation (Figure 16). 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). This inverse correlation was validated by a linear regression analysis.

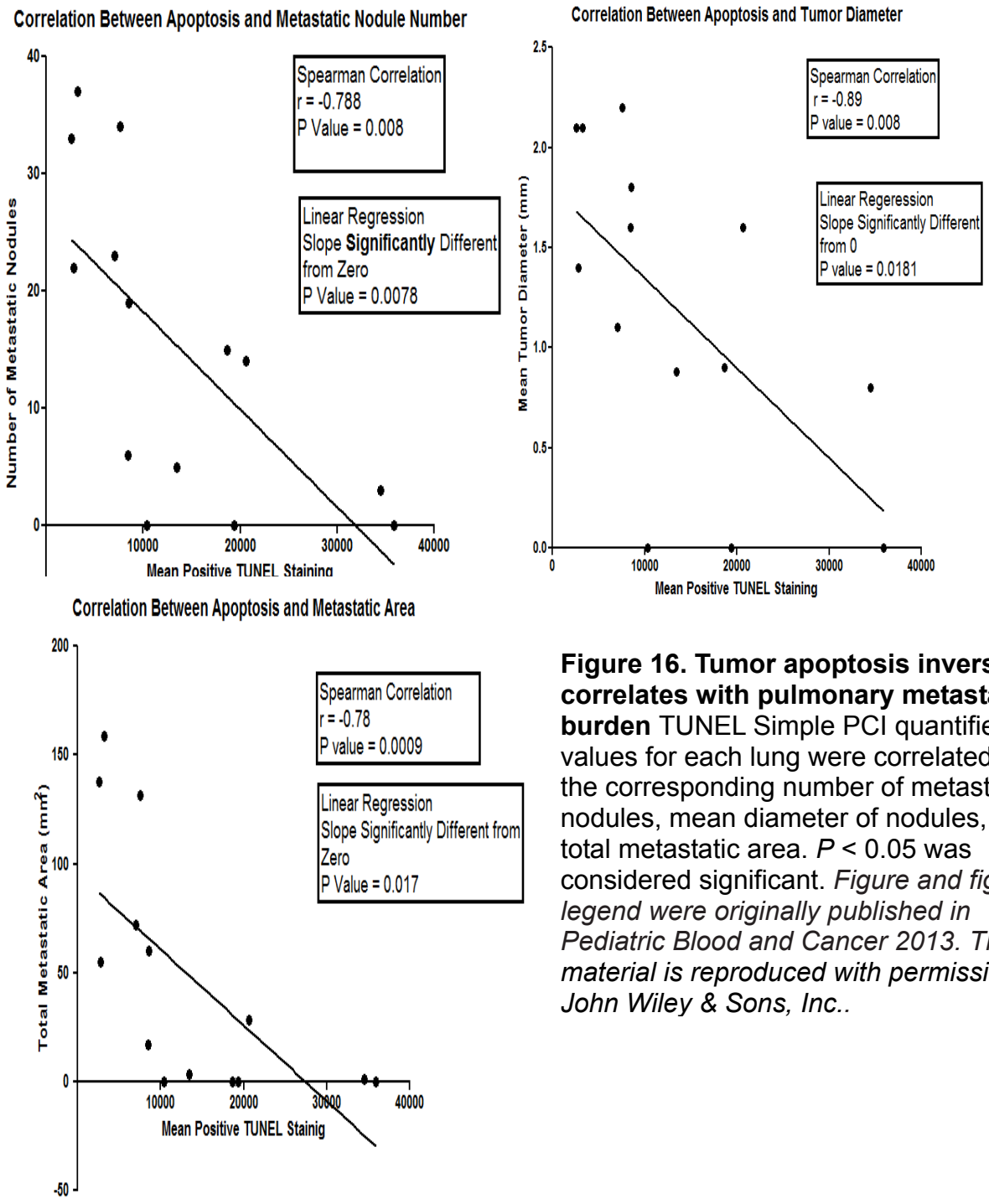


Figure 16. Tumor apoptosis inversely correlates with pulmonary metastatic burden TUNEL Simple PCI quantified values for each lung were correlated to the corresponding number of metastatic nodules, mean diameter of nodules, and total metastatic area. $P < 0.05$ was considered significant. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

SUMMARY

Combination therapy with aerosol IL-2 and NK cells significantly reduced pulmonary osteosarcoma metastasis, as measured by the number of tumors, the size of the metastatic nodules and by the metastatic infiltration of the lung. This was accompanied by a significant increase in tumor apoptosis. Tumor apoptosis inversely correlated with tumor number, tumor size, and metastatic infiltration. These results suggest that increased apoptosis is the mechanism behind the observed reduction in metastasis. This was expected, as NK cells kill by releasing cytotoxic granules that trigger the apoptotic pathway [28].

Giving aerosol IL-2 increases the efficacy of NK cell therapy against osteosarcoma lung metastasis. Our hypothesis was proven correct by the significant greater reduction in metastasis and increased tumor killing when aerosol IL-2 was given with NK cells versus to giving aerosol PBS with NK cells. Monotherapy with aerosol IL-2 slightly reduced tumor numbers and increased tumor killing, but to a much lesser degree.

RESULTS

Chapter 6

Aerosol IL-2 increased the NK cell infiltration of osteosarcoma metastasis

RATIONALE

We have shown that aerosol IL-2 increases the efficiency of NK cell therapy, as demonstrated by a reduction in osteosarcoma lung metastasis. This reduction is correlated with increased tumor apoptosis. Since aerosol IL-2 increases the presence of injected NK cells inside normal mouse lungs and NK cells kill primarily by activating tumor cell apoptosis [28], we hypothesized that aerosol IL-2 increased the number of NK cells inside the tumor nodules, thereby facilitating the physical interaction between the NK cells and their targets.

In this chapter we evaluated the infiltration of lung tumor nodules by human NK cells through the use of immunofluorescence staining. We also evaluated the presence of mouse NK cells in the same tumor nodules.

RESULTS

Aerosol IL-2 increased human NK cells in the metastatic osteosarcoma nodules in the lung

Fluorescent microscopy demonstrated increased human NKG2D staining in the lung metastasis from mice treated with aerosol IL-2 + human NK cells versus that from the mice treated with aerosol PBS + human NK cells ($P = 0.038$; Figure 17). Thus, aerosol IL-2 increases the contact between injected NK cells and their target tumor

cells.

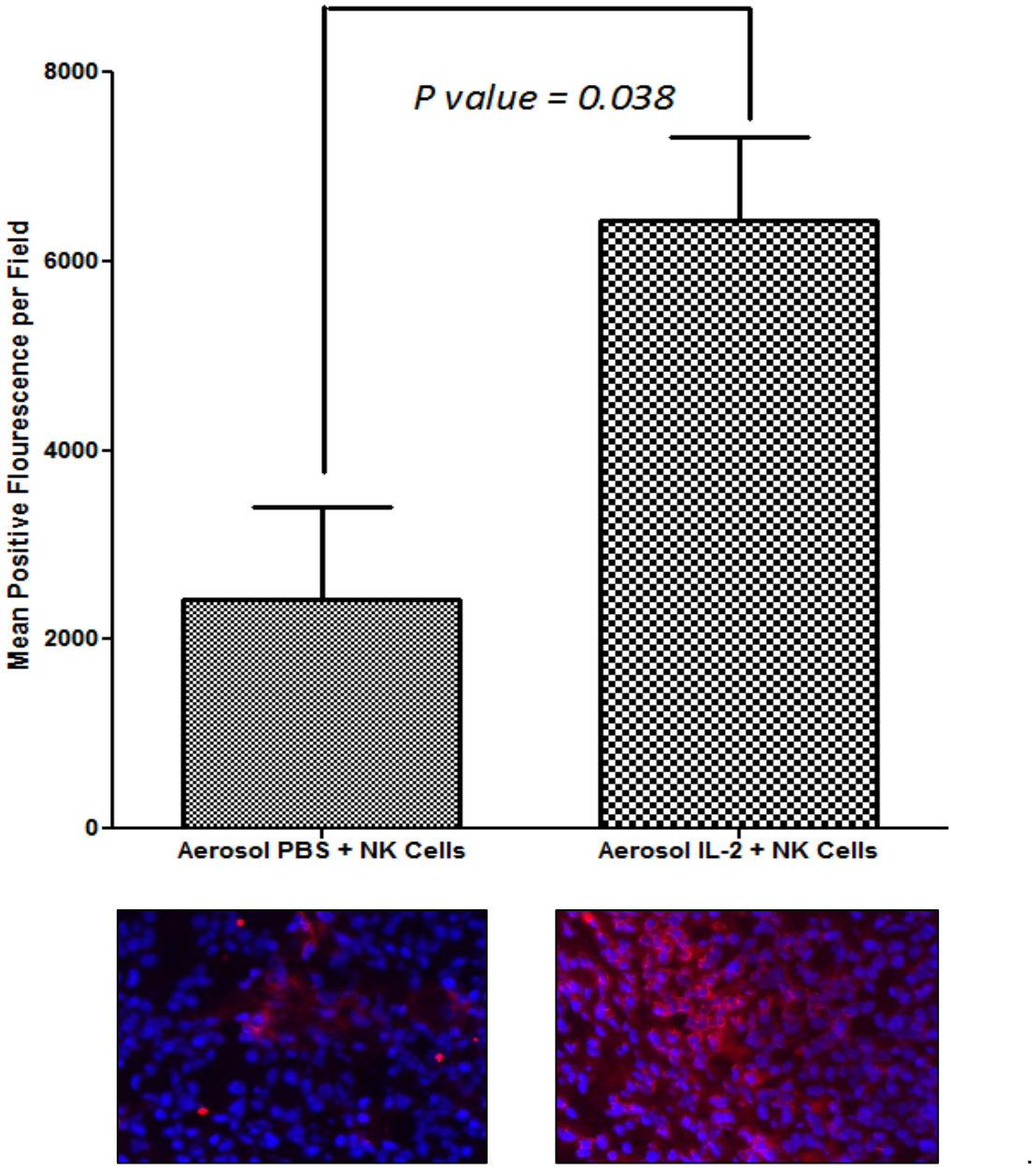


Figure 17. Aerosol IL-2 increased human NK cells in lung metastasis. Fluorescent microscopy was used to determine the NK cell infiltration in the lung using anti-human NKG2D (red). Cellular nuclei were identified with Hoechst33258 (blue). Mean positive fluorescence was quantified using the Simple PCI software in 5 random fields per section. $P < 0.05$ was considered significant. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Aerosol IL-2 increased mouse NK cells in the metastatic osteosarcoma nodules in the lung

Fluorescent microscopy demonstrated increased mouse NKp46 staining in the lung metastasis from mice treated with aerosol IL-2 versus that from mice treated with aerosol PBS (P = 0.05, Figure 18). This demonstrates that aerosol IL-2 therapy also increases the number of the mice's own NK cells inside the tumor. However the presence of mouse NK cells in the lung metastasis was much lower than the presence of human NK cells. Whether this contributes to aerosol IL-2's overall therapeutic effect is unknown. The number of mouse NK cells was also increased inside metastasis which were given aerosol IL-2 + human NK cells when compared to aerosol PBS treated mice (P = 0.013). However, there wasn't any statistically significant difference in the number of mouse NK cells present in the lung when comparing aerosol PBS + human NK cell-treated mice versus aerosol IL-2 treated mice and when comparing aerosol PBS + NK cell--treated mice versus aerosol IL-2 + human NK cell-treated mice (P = 0.1 and P = 0.23, respectively). NK cells are known to release a variety of cytokines and immune stimulators, such as INFγ and MIP-1α, both of which are potent macrophage activators [17]. Activated monocytes/macrophages can themselves secrete cytokines that activate NK cells [17]. There is a possibility that the injected human NK cells activate the mice's monocytes/macrophages, which would in turn stimulate the mice's own NK cells. There was no significant difference in the amount of mouse NK cells inside the lung metastasis when we compared aerosol IL-2 versus aerosol IL-2 + human NK cell treated mice (P =

0.2) and when we compared aerosol PBS versus aerosol PBS + human NK cell treated mice (P = 0.2).

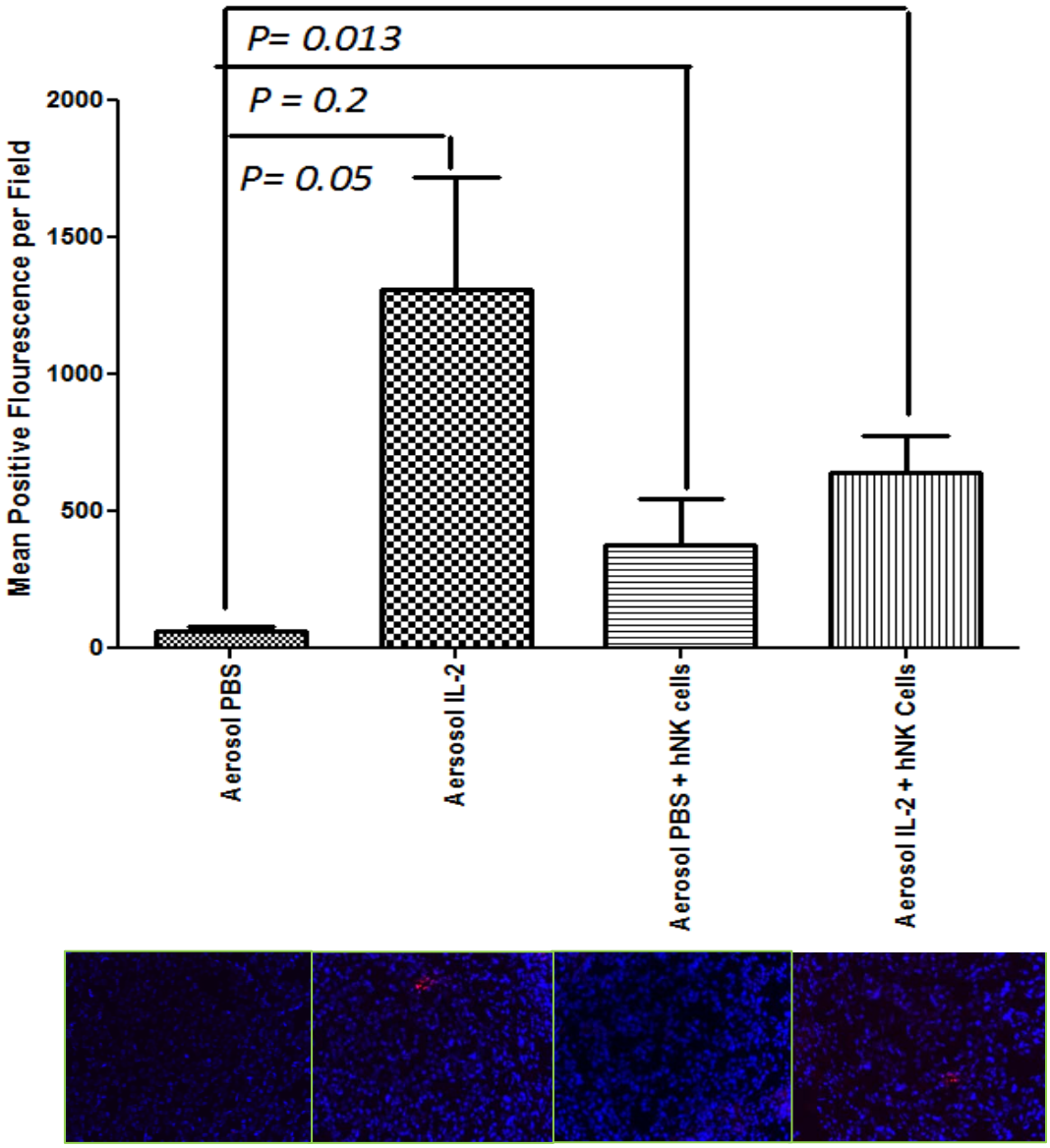


Figure 18. Aerosol IL-2 increased mouse NK cells in lung metastasis. Fluorescent microscopy was used to determine the NK cell infiltration in the lung using anti-mouse NKp46 (red). Cellular nuclei were identified with Hoechst33258 (blue). Mean positive fluorescence was quantified using the Simple PCI software in 5 random fields per section. $P < 0.05$ was considered significant.

SUMMARY

We have demonstrated that aerosol IL-2 increases the number of NK cells inside pulmonary osteosarcoma metastasis. This explains why combining NK cell therapy with aerosol IL-2 increases its effectiveness against osteosarcoma lung metastasis. A larger number of injected NK cells come into contact with their target tumor, increasing apoptosis and thus reducing lung metastasis. We have also demonstrated that aerosol IL-2 increases the number of mouse NK cells inside the tumor nodules. Whether direct killing of the human tumor cells by mouse NK cells contributes to aerosol IL-2's therapeutic efficacy is a matter of debate. Mouse NKG2D and human NKG2D recognize different ligands, with limited amino acid sequence homology among them [60]. However, even if mouse NK cells aren't directly responsible for the killing, they can still be activated by human IL-2 [72]. Consequently, they might in turn secrete other immune activating cytokines. For example, IL-2 activated NK cells secrete INFɣ, TNFα, MIP-1αβ, and GM-CSF, all of which stimulate macrophages [16]. Activated macrophages may be responsible for attacking the osteosarcoma cells.

RESULTS

Chapter 7

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

RATIONALE

We have demonstrated that aerosol IL-2 + NK cell therapy reduces pulmonary metastatic burden, as measured by a decrease in tumor numbers and size, as well as an increase in tumor apoptosis. One of the main goals for this investigation was to provide pre-clinical data to support moving forward with a clinical trial. To further test aerosol IL-2 + NK cell's potential as a therapeutic, we designed a study to determine whether treatment with aerosol IL-2 + NK cells increases overall survival.

3 million LM7 cells/mouse were injected intravenously through the tail vein of *nu/nu* mice. Once micro-metastasis was confirmed, treatment was initiated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells and was continued for 5 weeks. Aerosol treatment was given 3 times per week. 50 million human NK cells/mouse were injected through the tail vein 2 times per week. We then proceeded to determine the overall survival for the mice in the four treatment groups. We ended the survival study 162 days after starting treatment. Mice that had survived until this moment were euthanized. Long term survival was assessed from the first day of treatment until death.

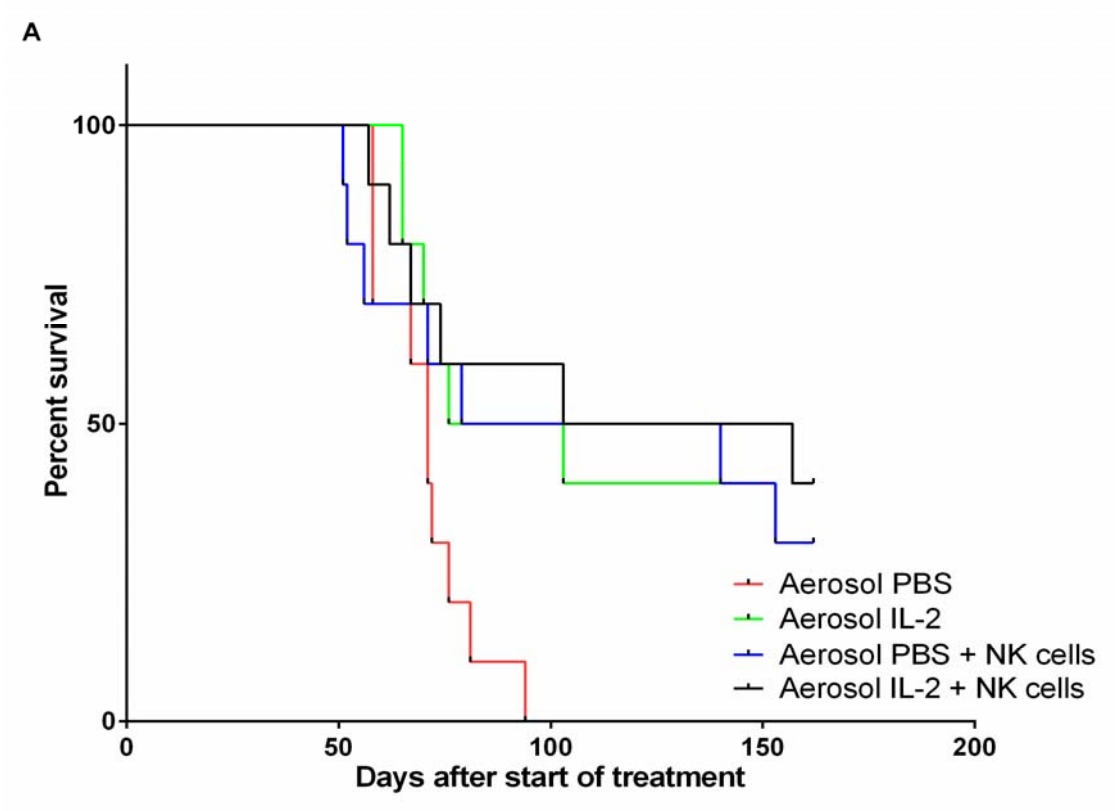
RESULTS

Aerosol IL-2 + NK cells increased the overall survival of mice with osteosarcoma lung metastasis

Aerosol IL-2 + NK cell therapy significantly increased the overall survival of mice with osteosarcoma lung metastasis (Figure 19B). Mice treated with aerosol PBS had a median survival of 71 days, while aerosol IL-2 + NK cell treated mice had a median survival of 130 days (P = 0.0153). By day 94 all the control mice were dead. 40% of the aerosol IL-2 + NK cell treated mice survived to the end of the study.

Treatment with aerosol IL-2 also increased the survival of mice with osteosarcoma lung metastasis (Figure 19C). Aerosol IL-2 increased the median survival from 71 days to 89 days (P = 0.03). Three aerosol IL-2-treated mice survived until the end of the study. The median survival of aerosol PBS + NK cell-treated mice was 109.5 days, higher than either the median survival of aerosol IL-2-treated or aerosol PBS-treated mice. Yet, because 3 aerosol PBS + NK cell-treated mice died very early in the study, the increase in overall survival was not significant when compared to control *(*P = 0.06; Figure 19D).

The median survival of mice treated aerosol IL-2 + NK cells was higher than those treated with aerosol IL-2 (Figure 19E) and those treated with aerosol PBS + NK cells (Figure 19F). However, the difference in overall survival was not significant (P = 0.7, P = 0.57, respectively). This was due to the increased life-span provided by aerosol PBS + NK cells and aerosol IL-2 treatment, though not as much as that provided by aerosol IL-2 + NK cells therapy.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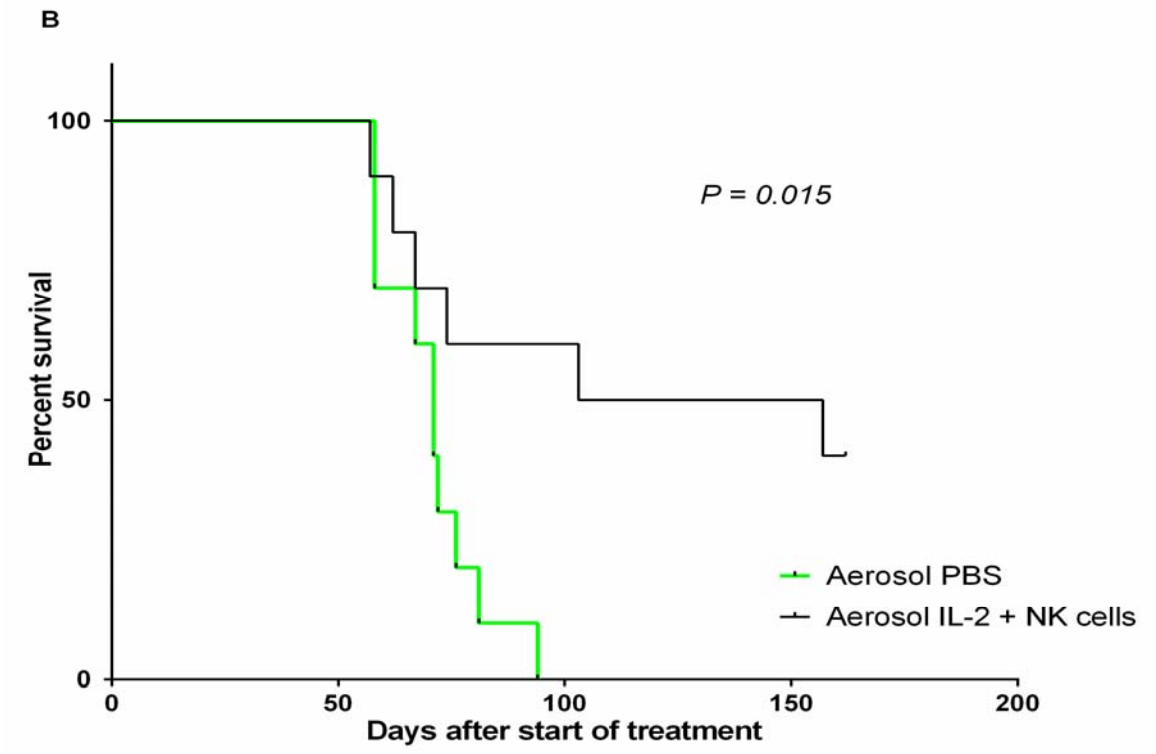
Aerosol IL-2 + NK cells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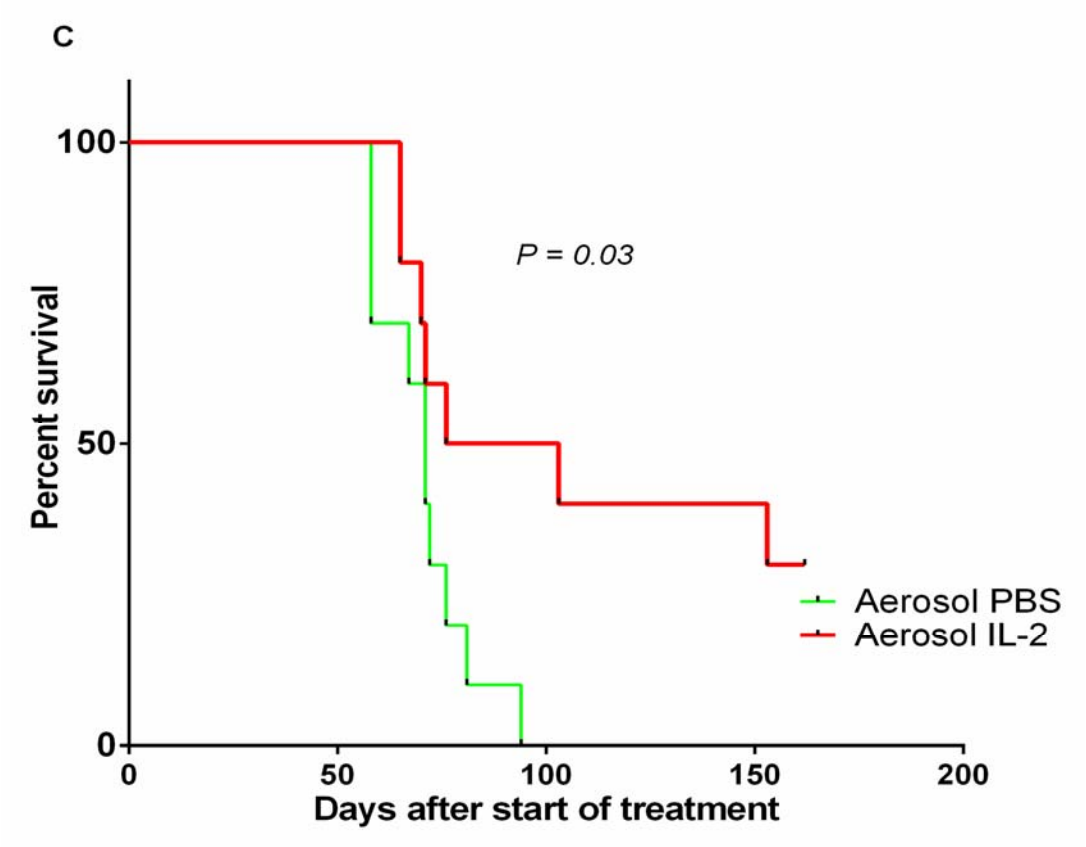
Days after start of treatment
Aerosol PBS
Aerosol IL-2 + NK cells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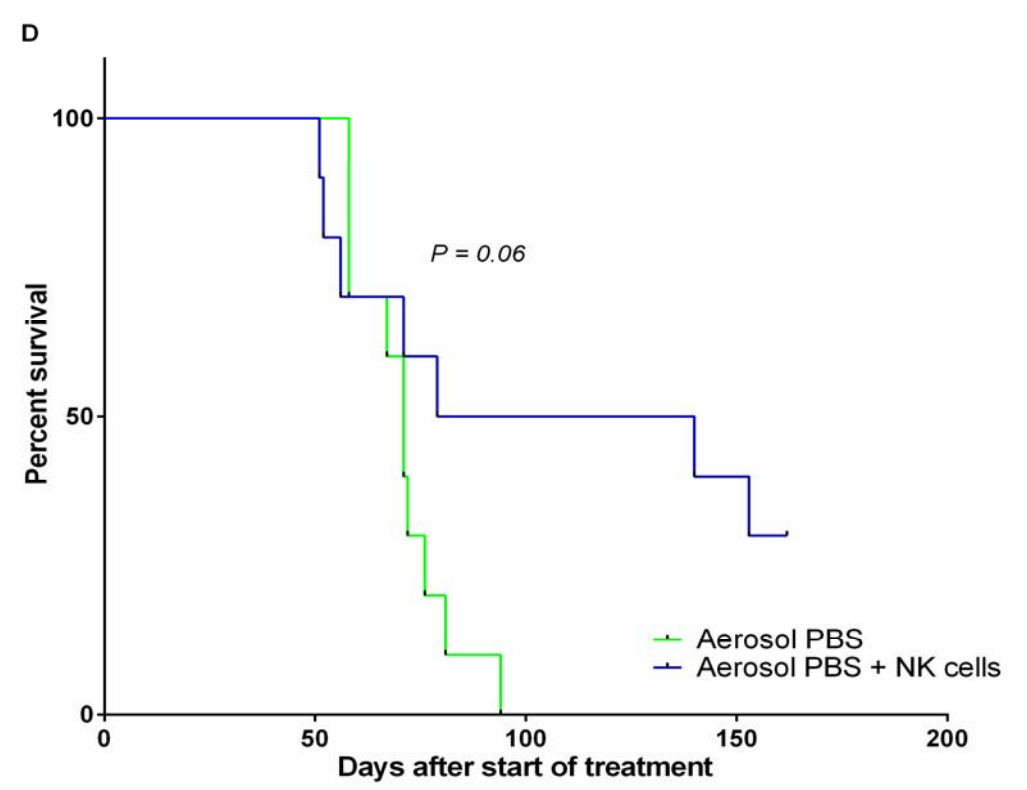
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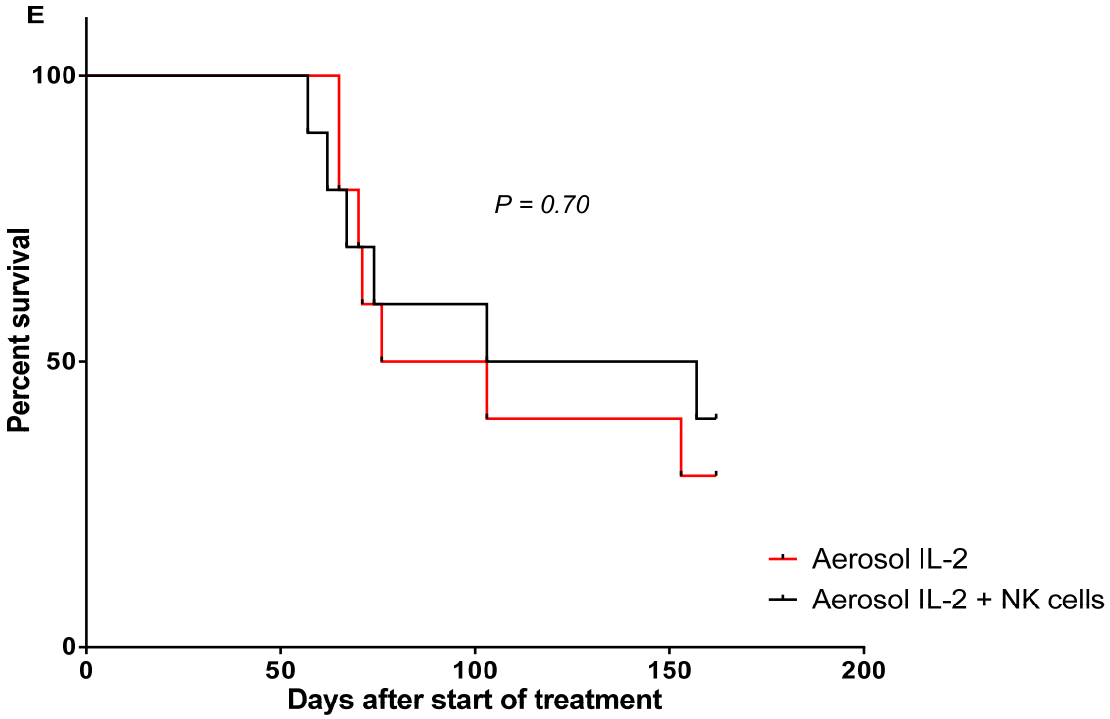
0
Percent survival
P = 0.06
Aerosol PBS
Aerosol PBS + NK cells
0
50
100
150
200
Days after start of treatment


E
100
50
0
Percent survival
P = 0.70
0
50
100
150
200
Days after start of treatment
Aerosol IL-2
Aerosol IL-2 + NK cells

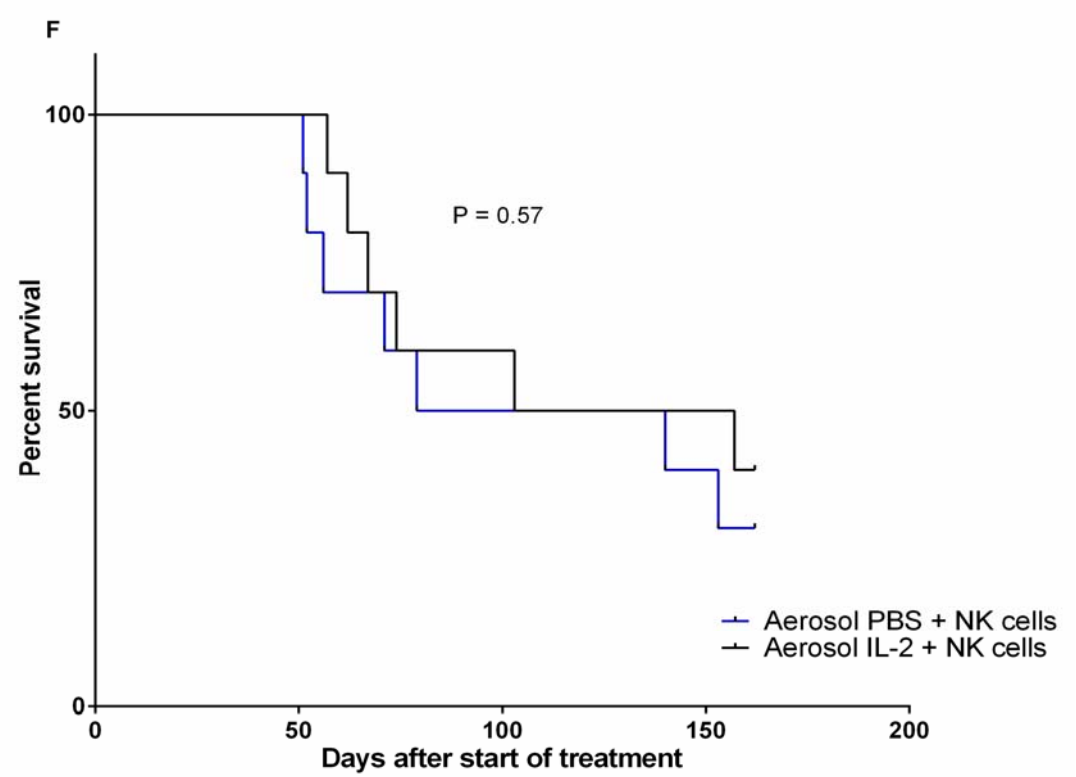


Figure 19. Effect of aerosol IL-2, NK cell therapy, and aerosol IL-2 + NK cells on the overall survival of mice with osteosarcoma lung metastasis. Nude mice were injected intravenously with LM7 cells. Therapy was initiated 6 weeks later and continued for 5 weeks. Mice were euthanized when they became moribund or after 162 days. **a.** Long-term survival was assessed from the first day of treatment. Overall survival of mice treated with aerosol IL-2 + NK cells (**b**), aerosol IL-2 (**c**) and aerosol PBS + NK cells (**d**) were compared to with those of aerosol PBS-treated mice. The overall survival of mice treated with aerosol IL-2 + NK cells was also compared with that of mice treated with aerosol IL-2 alone (**e**) or aerosol PBS + NK cells (**f**). The log-rank test was used to determine statistical significance. $P < 0.05$ was considered significant

As demonstrated in Figure 20, most of the mice that survived for 162 days did not have any visible metastasis present. Only one lung demonstrated the presence of metastasis. It belonged to a mouse treated with aerosol IL-2. This mouse only had one metastatic nodule with a diameter of less than1.3 mm. This demonstrates that treatment with aerosol IL-2, aerosol PBS + NK cells or aerosol

IL-2 + NK cells not only decreased tumor burden and increased survival, but were also capable of eliminating metastasis. We could say that macroscopically, 40% of the mice treated with aerosol IL-2 + NK cells were cured of the malignancy, while 30 % of the mice treated with aerosol PBS + NK cells and 20% of the mice treated with aerosol IL-2 were cured of malignancy.

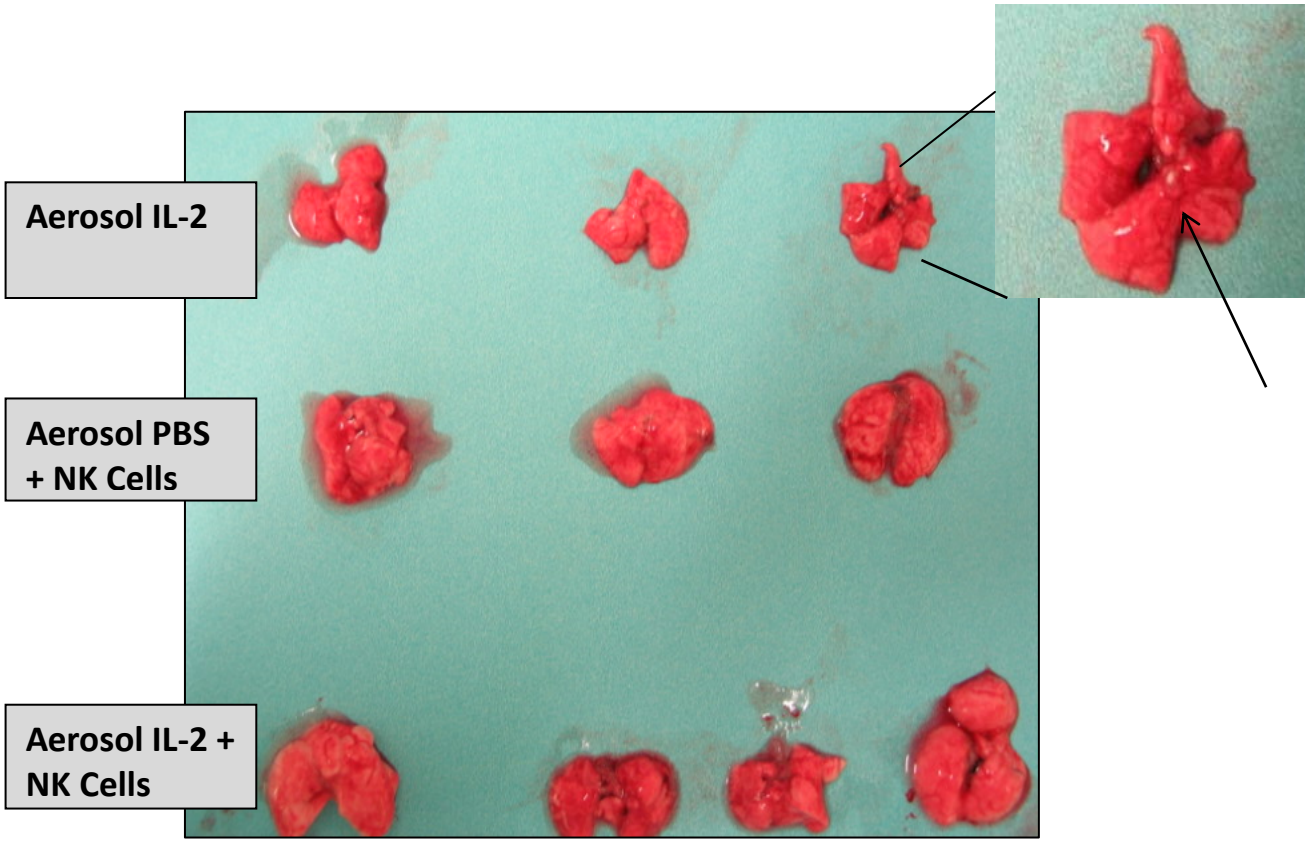


Figure 20: Representative picture of lung harvested from treated mice that were alive at the end of the study. Nude mice were injected i.v. with $3X10^6$ LM7 cells. Therapy was initiated 6 weeks later. Mice were treated with aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells for five weeks. Long-term survival was assessed from the first day treatment was initiated. Mice were euthanized when they became moribund and after 162 days.

SUMMARY

Our results demonstrate that aerosol IL-2 + NK cell therapy significantly increased the survival of mice with osteosarcoma lung metastasis. Treatment with aerosol IL-2 also significantly increased survival. However, the increase was less significant and to a lesser degree. Aerosol IL-2 + NK cell therapy increased the median survival from 71 days to 132 days. This median survival was higher than the survival seen in the other 2 treatment groups, though the difference was not significant. 40% of mice treated with aerosol IL-2 + NK cells survived the study at 162 days, with no macroscopic evidence of disease. 30% of mice treated with aerosol PBS + NK cells survived for 162 days, with no macroscopic evidence of disease. 30% of the mice treated with aerosol IL-2 survived for 162 days, with one of the mice demonstrating macroscopic evidence of disease. All the control mice had died by day 94.

The survival of the mice treated with aerosol IL-2 + NK cells was higher than that of the mice treated with aerosol IL-2 or NK cells alone. However, the difference in survival was not statistically significant. This was due to the ability of monotherapy with either aerosol IL-2 or NK cells to increase survival. In order to observe significance between the overall survivals between these treatment groups, a future experiment would be developed in which we extend the survival study beyond 162 days. As demonstrated in Figure 20, none of the lungs of the surviving mice treated with aerosol IL-2 + NK cells had evidence of macroscopic disease, while one mouse treated with aerosol IL-2 alone had macroscopic disease in the lung. In all probability, this mouse would have died if the study had been extended. A true survival study would be waiting until all of the mice in

each treatment group died, or until we could establish significant difference between the groups. If statistical significance could not be established, we would repeat the experiment starting treatment at earlier or later time points. The effectiveness of NK cell therapy is dependent on tumor size. Choosing a different time point to start treatment would mean treating lung nodules of greater or smaller sizes. This can translate into finding the difference in therapeutic efficacy between the three treatment modalities.

Chapter 8

Toxicity of aerosol IL-2 therapy

RATIONALE

One of the main reasons behind the use of aerosol IL-2 is to avoid the toxicities associated with systemic IL-2 treatment. The safety of aerosol IL-2 has been well documented in clinical trials [52, 55, 73]. Reversible airway irritation that causes non-productive coughing has been documented as the dose-limiting toxicity for aerosol IL-2 [55]. This side effect is much more tolerable compared to the high grades fevers, malaise, and fatigue normally associated with IL-2 treatment. In addition, it does not have the potential for the development of vascular leak syndrome, which has been reported to have caused respiratory failures in certain cases [74]. Patients report a much better quality of life, compared to systemic IL-2 treatments [52]. This lack of systemic toxicities is associated to very low IL-2 serum increases observed during aerosol treatment, with IL-2 levels peaking to just 1% of those observed after i.v. bolus injections [55, 75].

In this chapter, we describe how we evaluated aerosol IL-2 toxicity. Both *Nu/Nu* and *BALB/C* mice were used. *BALB/C* mice were used because of their immunocompetence. The lack of T-cells in *Nu/Nu* mice makes this model insufficient to represent aerosol IL-2-indued side effects. *Nu/Nu* mice had to be used to investigate NK cell associated toxicities because immunocompetent mice reject human NK cells.

RESULTS

Aerosol IL-2 does not cause acute or chronic toxicities in immunocompetent mice as evaluated by histological examination

BALB/C mice were treated with either aerosol IL-2 or aerosol PBS every other day for one week. Mice were sacrificed three days and one week after the start of the aerosol treatment to investigate acute toxicity. They were also sacrificed one month after the end of treatment. Lungs, spleens, livers, hearts and kidneys were fixed in formalin and embedded in paraffin. Corresponding H&E stained slides were analyzed with the help of Dr. John Stewart (Department of Pathology, MD Anderson Cancer Center). We did not find any histological evidence for acute or chronic inflammation, scarring or toxicity in the lungs, spleen, liver, heart or kidney of either the aerosol PBS or aerosol IL-2 treated mice (Figure 21).

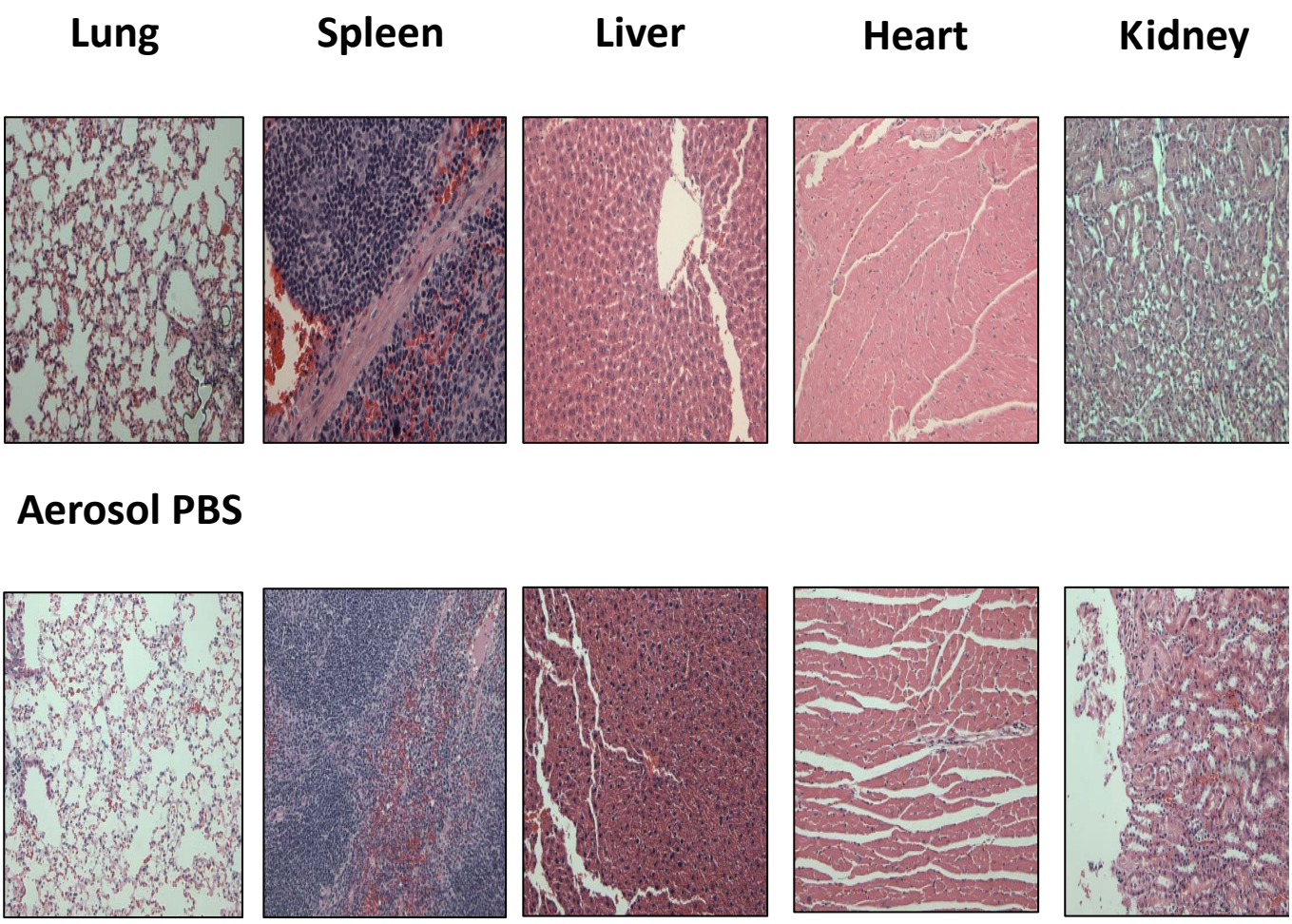


Figure 21: Aerosol IL-2 does not cause acute or chronic inflammation, scarring or toxicity. Representative H&E stained slides of lung, spleen, liver, heart and kidney of *BALB/C* mice treated with aerosol PBS or aerosol IL-2 for one week. Mice were euthanized either three days or one week after start of treatment, or one month after the end of treatment. Pictures shown were obtained from those mice sacrificed one month after the end of treatment. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Aerosol IL-2, NK cell therapy or aerosol IL-2 + NK cell therapy does not cause acute or chronic toxicities in *nu/nu* mice as evaluated by histological examination

Nu/Nu mice were treated for one week with aerosol PBS + NK cells or aerosol IL-2 + NK cells. Mice were euthanized one day, three days and one week after NK cell injection. Lungs, spleens, livers, hearts and kidneys were stained with H&E and analyzed with the help of Dr. John Stewart (Department of Pathology, MD Anderson Cancer Center). No histological evidence of acute inflammation, scarring or toxicity was found. *Nu/Nu* mice with LM7 metastasis were treated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells and aerosol IL-2 + NK for 5 weeks. Mice were euthanized at the 5^{th} week after start of treatment. Lungs, spleens, livers, hearts and kidneys were stained with H&E and analyzed with the help of Dr. John Stewart (Department of Pathology, MD Anderson Cancer Center). No histological evidence of chronic inflammation, scarring or toxicity was found.

Aerosol IL-2 therapy does not alter complete blood count or liver enzyme chemistry

The complete blood count [CBC] of mice with LM7 metastasis that had been treated with aerosol IL-2 or aerosol PBS for two and five weeks was determined (Figure 22 A, B). Aerosol IL-2 therapy did not alter CBC values. This suggests that aerosol IL-2 does not produce a significant systemic inflammation. Liver enzyme chemistry levels of the same mice treated for five weeks were also examined (Figure 22C). No significant changes in liver enzyme chemistry were found, suggesting aerosol IL-2 does not induce liver damage. Alkaline phosphatase was increased in aerosol PBS and aerosol IL-2 treated mice when compared to normal mouse basal levels. This was expected because alkaline

phosphatase levels are increased in patients with osteosarcoma, and is associated with worse prognosis [76].

A

	Aerosol IL-2	Aerosol PBS	P values	Normal Values
WBC (X10³/uL)	**8.3**+/- 2.6	**6.3** +/- 2.2	0.607088	2.1 - 7.1
RBC (X10³/uL)	**10.2** +/- 0.48	**10** +/-0.12	0.99123	7.4 - 9.9
HGB (g/dL)	16.1 +/- 1	**15.9** +/-0.44	0.712342	12.1 - 16.5
Platelets (X10³/uL)	**1216** +/-43	**1097** +/- 126.3	0.453315	659 - 1427
%Neut	**25.8** +/-5.2	**25.9** +/- 0.8	0.987794	7.4 - 25.9
%Lymp	**70** +/-5.55	**69** +/- 1	0.956285	60.8 - 85.0
% mono	1.75 +/-0.05	**2.1** +/- 0.3	0.861718	0.2 - 4.4
%eos	1.35 +/-0.15	**2** +/- 0.37	0.122537	0.0 - 13.0
%baso	0.15 +/-0.05	**0.17** +/- 0.067	0.854308	0.0 - 0.7
%Luc	1.1 +/-0.1	**0.6** +/- 0.3	0.224995	0.0 - 7.6

B

	Aerosol IL-2	Aerosol PBS	P values	Normal Values
WBC (X10³/uL)	**6.95** +/- 1.8	**7.8** +/- 1.3	0.738425	2.1 - 7.1
RBC (X10³/uL)	**10.45** +/- 0.45	**9.3** +/-0.03	0.239789	7.4 - 9.9
HGB (g/dL)	16.1 +/- 0.4	**15.5**+/-0.2	0.365371	12.1 - 16.5
Platelets (X10³/uL)	**596** +/-231	**1078** +/- 108.3	0.244185	659 - 1427
%Neut	**33.4** +/-2.5	**27.2** +/- 2.9	0.426165	7.4 - 25.9
%Lymp	**58.4**+/-7.15	**64** +/- 1.6	0.570168	60.8 - 85.0
% mono	2.3 +/-0.3	**3.8** +/- 1.3	0.312876	0.2 - 4.4
%eos	5.9 +/-3.7	**2** +/- 0.47	0.486989	0.0 - 13.0
%baso	0.35 +/-0.15	**0.4** +/- 0.1	0.810892	0.0 - 0.7
%Luc	2.5 +/-0.5	**1.25** +/- 0.05	0.305975	0.0 - 7.6

C

	Aerosol IL-2	Aerosol PBS	P values	Normal Values
AST	**94.5** +/- 7.5	**71** +/- 3.2	0.154397	54- 298(U/I)
ALT	**20** +/- 4	**27** +/-1.7	0.707965	17-77(U/I)
Alkaline Phosphatase	**1255** +/- 1199	**588**+/-388	0.676915	35-96(U/I)

Figure 22: Aerosol IL-2 does not alter CBC or Liver Enzyme Chemistry: Mice were treated with aerosol PBS or aerosol IL-2 for 2 (**A**) or 5 weeks (**B**) and their CBC was determined. **C**. Liver enzyme chemistry was determined after 5 weeks of treatment. Basal mouse levels for CBC and liver enzyme chemistry are shown. P< 0.05 was considered significant.

Serum IL-2 levels following aerosol IL-2 treatment

Serum IL-2 levels were measured in mice treated with aerosol IL-2 2 times per week for 2 and 5 weeks. Aerosol PBS treatment was used as the negative control. The positive control was serum from mice treated with a single dose of 20,000U IL-2 i.p, the dose normally used in therapeutic studies evaluating adoptive cellular therapy [77]. 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 the IL-2 levels measured in mice treated with a single dose of 20,000 U IL-2 i.p. (Figure 23) (*Originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc.)* These very low increases in serum IL-2 caused by aerosol IL-2 explain the lack of systemic side effects associated with aerosol IL-2, as well as the fact that aerosol IL-2 therapy did not increase the number of injected human NK cells in extra-pulmonary organs. It further strengthens the argument that aerosol IL-2 therapy is localized and organ-specific.

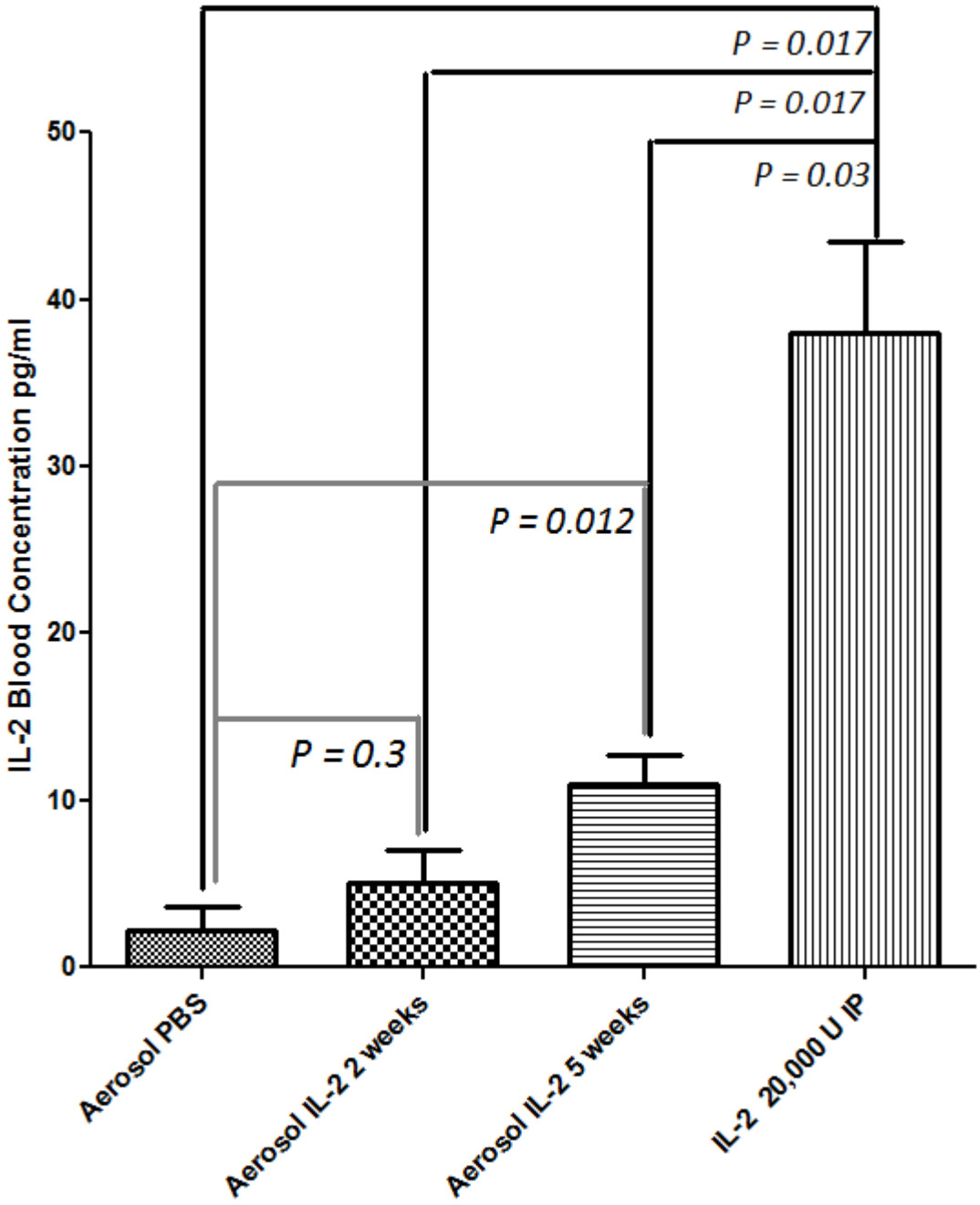


Figure 23: Serum IL-2 levels after aerosol IL-2 treatment: ELISA was used to determine serum IL-2 for mice treated with aerosol PBS, aerosol IL-2 for 2 weeks, aerosol IL-2 for 5 weeks and 1 dose of 20,000U IL-2 i.p. $P < 0.05$ was considered significant. *Figure and figure legend were originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc..*

Neither aerosol IL-2 nor NK cell therapy induces weight change in mice

To further corroborate that aerosol IL-2 is non-toxic, we monitored the weight of mice with LM7 lung metastasis treated for five weeks with either aerosol PBS, aerosol IL-2, aerosol PBS + NK cells, or aerosol IL-2 + NK cells. Weight change was monitored from the moment the treatment was initiated to one week after the treatment was stopped. As a control, we also monitored the weight change of a group of similarly aged mice for a period of 6 weeks. All mice experienced weight gain during a period of 6 weeks. However, there was no significant difference between the weights gained by the 5 groups (Figure 24). The mice that had no metastasis and received no treatment, gained an average of 2.07 g ± 0.6. This weight gain is not significantly different from the average weight gained by those with metastasis and treated with aerosol PBS (1.6 g ± 0.93; $P = 0.748$), aerosol IL-2 (2.62 g ± 0.8, $P = 0.564$), aerosol PBS + NK cells (2.47 g ± 1.37, $P = 0.773$) and aerosol IL-2 + NK cells (2.6 g ± 0.565; $P = 0.96$). In addition, the weight gain experienced by aerosol PBS treated mice was not significantly different from that experienced by mice treated with aerosol IL-2 ($P = 0.4$), aerosol PBS + NK cells ($P = 0.6$) and aerosol IL-2 + NK cells ($P = 0.4$).

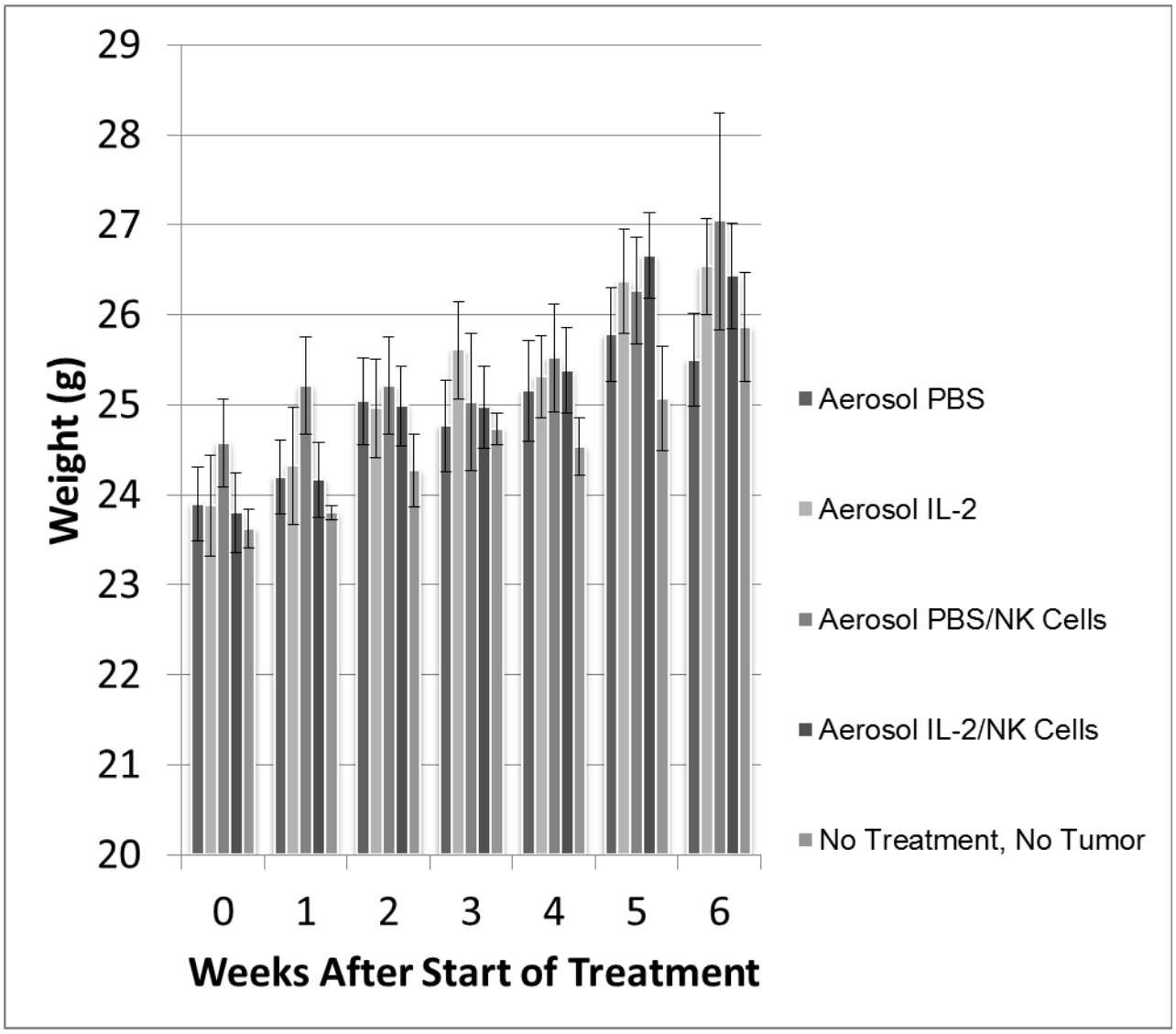


Figure 24. Weight gain during treatment. The weights of mice with LM7 metastasis and treated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells and aerosol IL-2 + NK cells for 5 weeks were monitored. The weight of untreated mice without lung metastasis was monitored as a control. $P<0.05$ was considered significant.

SUMMARY

In this chapter we established the safety of aerosol IL-2 therapy, NK cell therapy and aerosol IL-2 + NK cell therapy. Histological examination of the spleen, lung, liver, heart and kidney of mice treated with aerosol IL-2, aerosol

PBS + NK cells or aerosol IL-2 + NK cells showed no signs of acute or chronic inflammation, scaring, or toxicity. There weren't any treatment associated weight changes. Furthermore, aerosol IL-2 treatment did not alter the complete blood count or the liver enzyme chemistry. This establishes that aerosol IL-2 treatment is organ specific and does not induce an altered systemic inflammatory state. Systemic IL-2 is known to be able to cause leukopenia and rebound lymphocytosis [78], neither of which was suggested by the complete blood count. Other toxicities associated with systemic IL-2 that could be represented by a complete blood count include thrombocytopenia and anemia [79]. In addition, IL-2 can cause hepatic toxicity [79], which would present as increased serum liver enzymes. We also established that aerosol IL-2 can cause a small but significant increase in serum IL-2 levels if given for 5 weeks every other day. However, these levels are below those found in mice treated with intraperitoneal IL-2. This explains the lack of systemic side effects associated with aerosol IL-2 treatment and the reason why aerosol IL-2 increased NK cell numbers in the lung, but not in other organs. This suggests aerosol IL-2 activity is organ-specific.

Chapter 9

Discussion: Implications of Results and Future Directions

The 5-year survival rate for patients with osteosarcoma has plateaued at 60-70% using combination chemotherapy and surgery. With the exception of L-MTP-PE, no new drugs have been developed for either newly diagnosed or relapsed osteosarcoma in 20 years [10]. The primary cause of death is respiratory failure due to metastatic burden [2], as the lung is the most frequent site of metastatsis. Lung osteosarcoma metastasis has a very bad prognosis, with a 5 year survival of 33% [5]. Survival is even lower for relapsed patients. Less than 20% of relapse patients survive [6]. Moreover, salvage chemotherapy following relapse has been shown to slow disease progression but has no impact on long-term survival [80]. This implies the development of chemotherapy resistance. For this reason, we must look beyond traditional chemotherapeutics for the treatment of primary and recurrent osteosarcoma lung metastasis.

As mentioned before, L-MTP-PE is the first new agent approved for the treatment of osteosarcoma in the last 20 years [10]. Adding L-MTP-PE to standard chemotherapy for primary osteosarcoma increases the 6-year survival from 70 to 78% [9]. L-MTP-PE activates the tumoricidal properties of pulmonary macrophages and peripheral blood monocytes. The success of L-MTP-PE in increasing survival paves the way for the development of other immunotherapies to target osteosarcoma lung metastasis. Our laboratory previously showed that the intravenous injection of IL-11Rα-CAR$^+$ T-cells resulted in the regression of osteosarcoma lung metastases in a nude mouse model [11]. These findings further support the potential benefit of immunotherapy in the treatment of this malignancy. One of the drawbacks of IL-11Rα-CAR$^+$ T-cell therapy is the

collateral organ toxicity to those organs that express the IL-11 Rα. IL-11 Rα is expressed in the liver, stromal tissues of the GI tract, surface and gland epithelial cells, and on endothelial cells. By contrast, NK cells are a component of the innate immune system that recognize and kill malignant and virally infected cells but not normal host cells. Therefore the therapeutic: toxicity index is expected to be much higher.

NKG2D ligand expression in osteosarcoma

NKG2D ligands were highly expressed in both osteosarcoma cell lines (Figure 1) and patient tumor specimens (Figure 4). NKG2D is the major activating receptor on NK cells. The NKG2D ligand status determines the effectiveness of NK-cell based immunotherapies [58, 62]. These results suggest that osteosarcoma is a good target for NK cell therapy. Among the patient samples, a greater percentage of cells expressed NKG2D ligands in pulmonary metastases (77%) than in the primary tumors (57%). This validates the potential of NK cell therapy for relapsed disease in the lungs. NK killing assays further corroborated osteosarcoma's susceptibility to NK cells. It also demonstrated that the magnitude of in vitro killing correlated with the levels of NKG2D ligand expression (Figure 2). With the exception of KRIB cells, NK cytotoxicity against human osteosarcoma cells was 40 -50% at 1:1 E:T ratios. NK cytotoxicity against KRIB cells was near 10% at 1:1 E:T ratios. This reduced cytotoxicity correlated with the lower expression of NKG2D ligands found on KRIB cells when compared to the other cell lines (Figure 8). Conversely, NK cytotoxicity was significantly decreased when the NKG2D- NKG2D ligand interaction was blocked. This

demonstrates that NK-mediated killing of osteosarcoma is dependent on NKG2D ligand expression. It also suggests the effectiveness of NK cell therapy could be predicted based upon the patients' specific osteosarcoma NKG2D ligand expression.

Aerosol IL-2 increases NK cells in the lung

One of main purposes of using concomitant aerosol IL-2 was to increase the number of injected NK cells in the lung in order to facilitate their therapeutic potential against pulmonary osteosarcoma lung metastasis. We demonstrated that, while intravenous injection of fluorescent-labeled NK cells resulted in some localization in the lung, aerosol IL-2 significantly increased the number of NK cells in the lung three days post-injection (Figure 5). This increased localization of labeled NK cells was organ specific, as the number of NK cells was not increased in the liver, spleen, heart or kidney (Figure 6). In both aerosol PBS and aerosol IL-2 treated mice almost no human NK cells localized in the heart or kidney, while a very large amount were found present in the liver and spleen. These data is consistent with studies studying organ distribution of allogeneic NK cells in human patients [45]. Thirty minutes after transfusion, allogeneic NK cells aggregate in the lung. Two hours post transfusion they redistribute to the rest of the body, concentrating in the spleen, liver and bone marrow.

We determined the increase in the number of NK cells in the lung is caused by an increase in proliferation. Aerosol IL-2 significantly increases the percentage of proliferating NK cells in the lung (Figure 7), but not in the spleen or

liver (Figure 9). It slightly increases the proliferation of NK cells in the bone marrow 24 hours post injection, but not at 72 hours post injection (Figure 8). The increase in NK proliferation in the lung was greatest 72 hours after injection. This correlates to the time point in which we see the largest increase of NK cell numbers in the lung. This shows that the central site for aerosol IL-2 induced proliferation is the lung. On the other hand, intranasal respiratory virus infection induces NK proliferation in the bone marrow, not in the lung [71]. The increase of NK numbers in the lung is a result from migration from the bone marrow.

Aerosol IL-2 does not increase NK cell numbers or proliferation in extra pulmonary organs because aerosol IL-2 treatment for less than two weeks does not increase serum IL-2 levels (Figure 27). This suggests that the IL-2 from aerosol concentrates in the lung, with little spill over to the periphery.

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

Aerosol IL-2 also increases the number of injected NK cells present in the metastatic pulmonary nodules (Figure 17). We have already shown that NK killing of osteosarcoma is dependent on the E: T ratio (Figure 2). Having an increased presence of NK cells inside the tumor should translate into an increased therapeutic efficiency. NK-killing is mediated by the release of cytotoxic granules that trigger the apoptotic pathway [81]. The addition of aerosol IL-2 to NK cell therapy significantly increased the degree of apoptosis present in the tumor nodules (Figure 15). These increased levels of apoptosis correlated with decreased overall metastatic burden, as measured by tumor numbers and

tumor size (Figure 11- 14). Higher NK cell numbers contributed to increased tumor apoptosis, which translated into a better therapeutic outcome. However, aerosol IL-2's may also improve NK therapy by other mechanisms. IL-2 is known to increase the expression of NKG2D and NCR receptors. This makes NK cells more sensitive to activating ligands, which increases cytotoxicity and cytokine secretion [82]. IL-2 treatment can enhance NK adhesion and killing against weak targets, i.e., those who express low levels of activating ligands [83]. IL-2 also enhances F-actin reorganization in NK cells, necessary for cytotoxic granule release [84]. Aerosol IL-2 could have also increased the NK therapeutic efficacy by directly enhancing cytotoxicity in addition to increasing their proliferation.

Aerosol IL-2 also increased the number of mouse NK cells in metastatic pulmonary nodules (Figure 18). Whether this contributed to the therapeutic outcome is unknown. Monotherapy with aerosol IL-2 significantly increased tumor apoptosis (Figure 15). It also decreased tumor number and tumor size (Figure 11-14), though not significantly. However, it did increase mean survival (Figure 20). Mouse NKG2D and human NKG2D recognize different substrates, and there is little sequence homology among these [60]. It is unlikely that the observed increased tumor apoptosis was due to direct killing by mouse NK cells. However, mouse NK cells can be activated by human IL-2 [85]. Activated NK cells are known to secrete a variety of cytokines that influence other members of the immune system. For example, they release potent macrophage activators, such as INFγ and MIP-1α [17]. They can also kill M0 and M2 macrophages, but spare M1 macrophages [86]. This may suggest that IL-2 activated mouse NK

cells could have increased tumor apoptosis by enhancing the tumoricidal properties of the macrophage population in the lung. NK cells also interact with T cells and dendritic cells [16], but this interaction is not relevant in our investigation because our mouse model was athymic. NK cells are also able to kill endothelial cells [87]. The vasculature present in LM7 osteosarcoma metastasis is of mouse origin. Mouse tumor vasculature expresses NKG2D ligands [88]. This implies that tumor apoptosis could have been increased by a reduced blood flow caused by mouse NK-mediated killing of tumor vasculature.

Aerosol IL-2 + NK cell therapy increased overall survival

The enhanced tumor regression conferred by aerosol IL-2 + NK cell therapy rendered an increase in overall survival in mice with osteosarcoma lung metastasis (Figure 19B). Surprisingly, we also observed that monotherapy with aerosol IL-2 increased overall survival (Figure 19C). This may be due to an indirect activation of other immune cells in the lung. One plausible candidate may be an increased activation of lung macrophages. As we explained above, IL-2-activated mouse NK cells may have induced the polarization of macrophages into M1. Aerosol PBS + NK cell therapy also increased survival. However this increase in survival was not statistically significant. This was due to variability in the therapeutic efficacy seen with aerosol PBS + NK cell therapy. Some mice died early in the survival study, while others had an extended life-span. This suggests that the addition of aerosol IL-2 to NK cell therapy confers consistency in terms of its therapeutic effect.

Aerosol IL-2 Toxicity

One of the reasons we decided to use aerosol IL-2 to expand NK cells in the lung was to avoid the toxicities associated with systemic IL-2. IL-2 can cause a myriad of toxicities, including oliguria, hypotension, elevated liver enzymes, thrombocytopenia, and capillary-leak syndrome, which can lead to fluid retention, adult respiratory distress syndrome and myocardial infarction [49]. Histological examination of the lungs, spleen, liver, kidney and heart did not reveal any evidence of acute or chronic inflammation, edema, scarring, or other organ damage (Figure 21). CBC and serum enzyme levels were all within normal limits (Figure 22). There wasn't any weight associated changes in mice treated with aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells (Figure 24). Serum IL-2 levels following aerosol IL-2 therapy were well below those found in mice treated with intraperitoneal IL-2 (Figure 23). This explains the lack of toxicity. This suggests that aerosol therapy concentrates IL-2 in the lung, specifically activating the proliferation of the infused human NK cells in the lung (Figure 7), with very minimal spill-over into the periphery. This translates into the absence of systemic toxicities as well as the absence of IL-2-induced NK growth in the liver and spleen (Figure 9), and the minimal activation seen inside the bone marrow (Figure 8). Our results are consistent with clinical trials using aerosol IL-2, which demonstrate minimal toxicities [50, 51] and improved quality of life when compared to systemic IL-2 therapy [52].

Limitations of Study

We used human NK cells for our experiment partly due to the difficulty of isolating and expanding mouse NK cells *ex vivo*. For this reason, we used human osteosarcoma LM7 metastasis in a nude mouse model for our *in vivo* studies. Since nude mice do not have T cells, this had the added benefit of limiting any results demonstrating therapeutic effect to the interactions between aerosol IL-2, NK cells and the target tumor. However, the model does not present a complete picture of what would happen clinically. IL-2 is a potent activator of both cytotoxic T cells and regulatory T cells, of which the former may have a positive effect on therapy, while the later might limit it. Also, the model does not take into account the co-stimulatory relationships between cytotoxic T cells and NK cells [16], which could influence the effect of aerosol IL-2 + NK cell therapy. The lack of T cells may limit an accurate picture of the toxicity of aerosol IL-2, as IL-2 systemic side effects could be attributed to T cell activation. However, we addressed this concern by treating BALB/C mice with aerosol IL-2 in our toxicity studies (Figure 21) and finding no histological evidence of acute or chronic inflammation, scarring or damage in sections of lung, spleen, heart, liver or kidney.

Conclusion

There is a need for the development of new therapeutics for patients with osteosarcoma lung metastasis. In the last 20 years, mifamurtide has been the only new therapeutic that has been approved for the treatment of osteosarcoma

[10].Our data has shown that the aerosol delivery of IL-2 is an effective way to induce selective migration and expansion of NK cells in the lungs. Aerosol IL-2 also increased human NK cell numbers within lung tumor nodules and increased tumor cell apoptosis. This translated into reduced pulmonary metastasis and increased survival. Combining aerosol IL-2 therapy with NK cells increased the therapetic efficiency of NK cell therapy without causing systemic toxicity. Since the safety and tolerability of aerosol IL-2 have already been documented in several different clinical trials [50, 51], this organ-specific cytokine delivery concept can be exploited to target selective NK cell migration, expansion, and activation in the lung. This combination therapy can therefore serve as a new therapeutic approach for patients with relapsed and unresponsive osteosarcoma lung metastases.

FUTURE DIRECTIONS

Translation into Clinical Trials

One of the main purposes of this investigation was to provide pre-clinical data testing a novel therapeutic approach for the treatment of osteosarcoma lung metastasis with the possible goal of moving forward with a clinical trial. Currently a Phase I/II clinical trial is being run in MD Anderson with the purpose of determining the highest tolerable dose of aerosol IL-2 and its safety in the context of patients with lung metastasis. Among those to be treated with aerosol IL-2 are patients with osteosarcoma lung metastasis. Dr. Nancy Gordon (Division of Pediatrics, The University of Texas MD Anderson Cancer Center) from Dr.

Kleinerman's laboratory is coordinating this effort. Serum concentrations of IL-2 will be reported to determine if any peripheral spill-over occurs. Peripheral absolute lymphocyte counts will also be recorded. Early lymphocyte recovery after chemotherapy has been associated with improved prognosis in pediatric patients with osteosarcoma [89]. We would attempt to correlate the effectiveness of aerosol IL-2 therapy with absolute lymphocyte counts. Lung tumor biopsies would be collected before and after aerosol IL-2 treatment. Using immunohistochemistry, we will determine if aerosol IL-2 increases NK cell infiltration within the patient lung nodules and we will correlate this data with the degree of apoptosis and/or necrosis within the metastatic nodules. If any objective response is observed in this clinical trial, we would want to determine if it correlates with increased NK cell tumor infiltration and tumor apoptosis. Any positive correlation would give impetus to moving forward with a Phase I clinical trial examining the use of aerosol IL-2 and NK cell therapy.

The purpose of a phase I clinical trial involving the use of aerosol IL-2 + NK cells will be to determine the highest tolerable dose and safety of *ex vivo* expanded [39] allogeneic KIR-mismatched NK cells given in combination with aerosol IL-2 therapy in the context of patients with osteosarcoma lung metastasis. The dose of aerosol IL-2 to be given would be determined by the above mentioned clinical trial. To our knowledge, there have been no investigations that have reported a peak tolerable dose for infused NK cells. The highest dose of NK cells to have been infused into patients has been $4.7 \times 10^{10} \pm 2.1 \times 10^{10}$ *ex vivo* expanded autologous NK cells. No toxicities were reported in

this study [37]. The most toxic adverse effects associated with NK cell infusions have been Grade I and II pruritus, rigors and muscle aches using 2 X 10^7/kg NK-enriched KIR mismatched cells [90]. In our investigation we injected 5 X 10^7 NK cells per mouse two times per week for 5 weeks. Considering *nu/nu* mice weigh between 20 and 30 grams, the corresponding NK cell dose should be between 1.7 X 10^9 - 2.5 X 10^9 NK cells/kg for a human patient 2 times per week for 5 weeks. Since such high numbers of NK cells have never been infused into patients before, a gradual increase in NK cell infusions should be attempted in order to determine the highest tolerable dose. Patients would be treated with NK cell therapy with or without concomitant aerosol IL-2. Therapeutic effect, as well as possible NK toxicities, could be exacerbated by the addition of aerosol IL-2. If throughout the trial we observe that the addition aerosol IL-2 confers an additional therapeutic benefit, we would add aerosol IL-2 to those patients receiving NK cell therapy alone. Tumor biopsies would be obtained as well as blood samples before and after treatment. We would want to determine the degree of NK tumor infiltration as well as tumor apoptosis. We would want to determine if increased NK tumor infiltration, increased absolute lymphocyte counts and increased peripheral NK cells correlate with increased tumor apoptosis and with objective response, if any is seen.

After determining the highest tolerable dose and the safety of NK cell therapy, a Phase II clinical trial would be designed. If a peak tolerable dose of NK cells cannot be established, then the dose to be used in this trial will correspond

to the dose associated with the highest objective response and/or with the highest degree of tumor apoptosis.

As demonstrated by killing assays (Figure 2), NK cell cytotoxicity increases with increasing E:T ratio. An E:T of at least 5 is required for maximum efficiency. To increase therapeutic efficiency, a Phase II clinical trial should be designed so that aerosol IL-2 + NK cell therapy is given in the context of minimal residual disease (MRD) or after the surgical excision of the metastatic lesions. We would not expect NK cell therapy to successfully destroy a bulky tumor with a large surface area. We believe NK cell therapy would be more effective against microscopic metastatic foci. Treatment duration would be dependent on the Phase I trial that is currently being undertaken to determine the safety of aerosol IL-2 therapy and our envisioned Phase I trial that would determine the highest tolerable dose of NK cells given in combination with aerosol IL-2. We hope that aerosol IL-2 + NK cell therapy will increase the 5-year survival of patients with osteosarcoma lung metastasis beyond 33% [5].

Soluble NKG2D ligand concentration in patients with osteosarcoma

Our data for patient specimens demonstrated that NKG2D ligand expression was higher in osteosarcoma lung metastasis than in primary tumors (Figure 4). This seems to be counterintuitive in terms of evasion from NK immunosurveillance. Decreased expression of NKG2D ligands on colorectal cancer correlates with tumor stage progression, with the lowest expression associated with stage IV [91]. One of the mechanisms by which tumors with

elevated NKG2D ligand expression can evade NK recognition is by shedding NKG2D ligands. Indeed, serum soluble MICA is increased in metastasis [92]. In melanoma, elevated serum soluble ULBP2 levels correlate with a poorer prognosis [93].

To asses if osteosarcoma lung metastasis is associated with increased NKG2D ligand shedding, we would measure the serum concentration of soluble NKG2D ligands in patients with osteosarcoma lung metastasis and compare these values to serum concentrations from patients with primary osteosarcoma and health patient serum. We would corroborate these data with *in vitro* studies measuring the concentration of soluble NKG2D ligands in the culture media from human osteosarcoma cells. We would compare the secretion of NKG2D ligands by osteosarcoma cell lines derived from patient pulmonary metastasis and the secretion by cell lines derived from primary osteosarcoma. If the results demonstrate that osteosarcoma metastasis increases soluble NKG2D ligand expression, this could explain how metastatic osteosarcoma cells evade NK immunosurveillance even when they have high surface expression of NKG2D ligands.

Matrix Metalloproteinase 9 (MMP9) is responsible for MICA shedding in osteosarcoma. Inhibiting MMP9 prevents MICA shedding and increases osteosarcoma susceptibility to NK cytotoxicity [94]. We hypothesize that the addition of a MMP9 inhibitor would enhance NK cell therapy against osteosarcoma lung metastasis. To test this hypothesis we would first chose a human osteosarcoma cell line derived from a patient with pulmonary metastasis

capable of secreting high levels of soluble MICA. LM7 would not necessarily secrete high levels of soluble MICA because it was derived from the primary human osteosarcoma cell line SAOS-2 after becoming metastatic by passage through the lungs of *Nu/Nu* mice [64]. The ability to secrete soluble human NKG2D ligands would not have provided LM7cells with a survival advantage in a tumor micro-environment with mouse NK cells because mouse NKG2D does not recognize human NKG2D ligands [60]. Once we confirm metastasis, we would treat these mice with NK cells that have been expanded *ex vivo* with IL-2 and genetically modified K562 with membrane-bound IL-21 and IL-15 [39] in combination with or without a MMP9 inhibitor. We would use mice treated with MMP9 inhibitor alone or without treatment as negative controls. Since *ex vivo* expansion growth increases NK cytotoxicity, numbers and life-span, we might not discover any increased therapeutic effect by the addition of MMP9 inhibitors. Increased cytotoxicity and increased NK life-span may overcome the inhibitory effect mediated by soluble NKG2D ligands. To corroborate this, we would repeat this experiment with freshly isolated NK cells. Since aerosol IL-2 increases NK cell proliferation in the lung, we would also want to determine if combination NK cell and aerosol IL-2 treatment can overcome any inhibition provided by soluble NKG2D ligands. We would design an experiment similar to the one described above with the addition of aerosol IL-2 to the NK cell therapy. If the addition of MMP9 inhibitor does not significantly improve the therapeutic efficacy of aerosol IL-2 + NK cell therapy, but does improve therapeutic efficacy when added to

aerosol PBS + NK cell therapy, we may be able to conclude that co-treatment with aerosol IL-2 overcomes the inhibition that is induced by MICA shedding.

Pharmacological upregulation of NKG2D ligands

If aerosol IL-2 + NK cell therapy is to be used in clinical trials, we should consider the fact that a substantial number of patients with osteosarcoma lung metastasis have been exposed to chemotherapeutical agents. Chemotherapy may alter the NKG2D ligand expression in osteosarcoma lung metastasis. However, since NKG2D ligands are upregulated in response to cellular stress [23], chemotherapy may actually aid NK cell therapy.

Cisplatin, doxorubicin, and high-dose methotrexate with leucovorin are the standard chemotherapeutical agents used against osteosarcoma lung metastasis [6]. Doxorubicin has been shown to increase NKG2D ligand expression on multiple myeloma cells and to enhance their susceptibility to NK cytotoxicity [95]. To determine if the standard chemotherapy used against osteosarcoma increases NKG2D ligand expression, we would obtain patient specimens from osteosarcoma lung metastasis before and after chemotherapy and we would then do immunohistochemistry staining for NKG2d ligands. We would supplement these data with *in vitro* studies comparing the NKG2D ligand expression of osteosarcoma cells before and after treatment with the above mentioned chemotherapeutical agents. NK killing assays would also be performed to determine if chemotherapeutic treatment of osteosarcoma cells enhances NK killing. If our *in vitro* data and our patient sample data demonstrate

that standard chemotherapy against osteosarcoma lung metastasis increases the expression of NKG2D ligands, we should consider giving NK cell therapy shortly after chemotherapy has been administered to patients.

We could also investigate alternate chemotherapeutical agents that would enhance osteosarcoma NKG2D ligand expression and potentially enhance NK cell therapy. For example, our laboratory has successfully demonstrated that aerosol gemcitabine treatment of osteosarcoma lung metastasis significantly inhibits its growth and increases tumor Fas expression [96-98]. Gemcitabine is known to increase the expression of MICA/B in hepatocellular carcinoma and to also increase their susceptibility to cytokine-activated killer cells [99]. We would design an investigation in order to determine if gemcitabine increases the expression of NKG2D ligands in osteosarcoma and if this translates into a greater therapeutic efficacy when using NK cell therapy. First, we would determine if gemcitabine increase NKG2D ligands *in vitro* on various osteosarcoma cell lines. Next, we would determine if the expression of NKG2D ligands in osteosarcoma lung metastasis from mice treated with aerosol gemcitabine is increased. We could use a *Nu/Nu* mice model with human osteosarcoma lung metastasis and/or BALB/C mice model with mouse osteosarcoma lung metastasis. If we decided to use the BALB/C model, we would need to test for the expression of mouse NKG2D ligands. If our results demonstrate gemcitabine-induced NKG2D ligand increase, we would perform a therapeutic study combining NK cell therapy with aerosol gemcitabine.

HDAC inhibitors are promising agents with the potential to increase the expression of NKG2D ligands in cancer. Valproic acid increases the expression of NKG2D ligands on myeloma [100], ovarian cancer [101], Ewing sarcoma [102], hepatoma [103], and AML[104], as well as increasing their susceptibility to NK-mediated cytotoxicity. Valproic acid has also been reported to increase the expression of MICA/B in osteosarcoma cell, without increasing its soluble form, leading to an increased susceptibility to NK killing [105]. In our laboratory, we've had great success with the use of another HDAC inhibitor, MS-275. Our laboratory has shown that MS-275 increases the Fas expression in osteosarcoma and induces the regression of osteosarcoma lung metastasis [106-108]. Graduate student Simin Kiany is currently investigating the use of MS-275 to increase the expression of NKG2D ligands in osteosarcoma and using it in combination with NK cell therapy to treat osteosarcoma lung metastasis in a *Nu/Nu* mouse model.

Increasing NK homing toward osteosarcoma metastatic nodules in the lung

Aerosol IL-2 increases the number (Figure 5) and proliferation (Figure of 7) of infused NK cells in the lung, leading to increased NK cell retention in tumor nodules (Figure 16), which in turn increases tumor apoptosis (Figure 14) and therapeutic efficacy. Yet, a large proportion of infused NK cells still propagate toward the spleen and liver (Figure 6). Finding a way to increase the retention of infused NK cells in the lung or to induce their migration from extra-pulmonary organs could potentially improve the therapeutic efficacy of NK cell therapy. Our

laboratory has started investigating the role chemokines and chemokine receptors play in NK cell migration toward tumor locations.

The study of the chemokine receptor CX3CR and its ligands shows great promise. CXC3CR is a chemokine receptor that is expressed on T cells and activated NK cells [109]. Pre-clinical studies report that stimulating the expression of CX3CR ligands causes lung cancer regression. Antitumor activity was mediated by an increased T cells and NK cell tumor infiltration [110]. The clearance of YAC-1 cells in the lung of C57BL/6 is mediated by NK cells and is dependent on the interaction between CXC3CR and the chemokine CX3CL1[111]. The CXC3CL1/CXC3CR chemokine/receptor axis has also been implicated in the eradication of neuroblastoma metastases by T cells and NK cells [112]. Gene therapy using aerosolized liposome encapsulated CX3CL1-encoding plasmids significantly reduced mouse osteosarcoma lung metastasis [113]. We can design a pre-clinical trial in which aerosolized gene therapy with CX3CL1-encoding plasmids is combined with aerosol IL-2 + NK cells in order to maximize the killing of osteosarcoma lung metastasis. Aerosol IL-2 would increase the numbers of NK cells in the lung, while CX3CL1 transduction of metastatic osteosarcoma cells would enhance the migration of these NK cells toward the tumor nodules facilitating tumor cell killing. It could also provide the added benefit of attracting the NK cells that would normally propagate toward the liver and spleen back toward the tumor sites. For maximum effect, we could specifically select and expand NK cells with high expression of CXC3CR.

Another mechanism of exploiting NK chemotaxis toward osteosarcoma would be to investigate the normal chemokine expression of metastatic osteosarcoma cell lines. We would then determine which of these chemokines are more effective in attracting NK cells. We would then selectively isolate and expand NK cells with these specific receptors for these chemokines from buffy coats for subsequent adoptive *ex-vivo* expanded NK cell therapy. Dr. Gangxiong Huang of Dr. Kleinerman's laboratory is currently working on this project. Recently Somanchi *et al* [114] engineered a way to successfully transfer the chemokine receptor CCR7 to *ex vivo* expanded NK cells by incubation with CCR7-expressing K562s. The plasma membranes of these NK cells acquired CCR7 via trogocytosis. These NK cells could express CCR7 for 72 hours. This transient expression of CCR7 successfully enhanced the migration of NK cells toward the lymph nodes of athymic nude mice. We could select specific chemokines that are actively expressed in osteosarcoma cells of metastatic origin. We would design a pre-clinical study in which ex vivo expanded NK cells are incubated with K562s expressing the chemokine receptor or receptors for these chemokines. This could potentially enhance the migration of NK cells toward osteosarcoma metastatic cells in the lung and enhance therapeutic effect.

Exploiting the cross-talk between Macrophages and NK cells

Preliminary unpublished data from our laboratory demonstrates increased macrophage infiltration of lung osteosarcoma metastasis in *Nu/Nu* mice treated with aerosol IL-2. *Nu/Nu* lack T cells and mouse NKG2D does not recognize human NKG2D ligands [60]. Increased macrophage infiltration could explain the

reason we see a therapeutic effect in *Nu/Nu* mice with human osteosarcoma metastasis that have been treated with aerosol IL-2 alone. Aerosol IL-2 may activate mouse NK cells in the lung, and this activated NK cells might subsequently activate lung macrophages. NK cells are known to secrete a variety of cytokines that can activate monocytes/macrophages, including INFɣ, TNFα, MIP-1αβ, and GM-CSF. Macrophages in turn secrete IL-12 and IL-18 to activate NK cells [16]. Physical contact between macrophages and NK cells increases the expression of NKG2D [115]. Activated NK cells have the ability to shift macrophage polarization toward M1. They do this by killing M0 and M2 macrophages, but sparring M1 macrophages [86]. This suggests an important role for macrophages in NK-mediated tumor regression.

We could test this hypothesis by first determining the state of polarization in the macrophage infiltrate of aerosol PBS-treated, aerosol IL-2-treated, aerosol PBS + NK cell-treated and aerosol IL-2 + NK cell-treated LM7 osteosarcoma lung metastasis in *Nu/Nu* mice. Markers for M1 polarization are CD68/HLA-DR, and those for M2 are CD68/CD163 [116]. If NK-mediated activation of macrophages to a M1 polarized state is associated with NK-mediated tumor regression, we would see an increased M1 polarized state in the lung metastasis that have been treated with aerosol IL-2, aerosol PBS + NK cells and aerosol IL-2 + NK cells when compared with aerosol PBS treatment. NK cell-macrophage crosstalk is dependent on both cytokine secretion and physical contact. Human NK cells might still activate mouse macrophages by the secretion of certain cytokines, but the activation induced by cell-to-cell contact may be lost. For example, one of the

ways human macrophages activate human NK cells is by the expression of MICA [117]. This would not be possible between human NK cells and mouse macrophages, as mouse macrophages would lack human NKG2D ligands. For this reason, we may not see much difference in terms of M1 polarization when we compare aerosol IL-2, aerosol PBS + NK cell and aerosol IL-2 + NK cell-treated tumors.

If macrophage activation is important for NK-mediated tumor regression, we could potentially enhance this regression by the addition of macrophage activators. As mentioned before, L-MTP-PE has been the only new therapeutic that has improved survival in osteosarcoma patients [10]. Since L-MTP-PE is a direct macrophage activator and since NK cells and macrophages can co-stimulate each other, a combination therapy with aerosol IL-2 + L-MTP-PE + NK cells could be a novel and effective therapeutic regiment against osteosarcoma lung metastasis.

Aerosol therapy with other NK activating cytokines

In this investigation we used aerosol IL-2 for the stimulation of NK cells in the lung due to IL-2's well known effect on NK cells. However, there are a variety of other cytokines that are known to stimulate NK cells. IL-15 is another well-known activator of NK cells and it shares structural similarities with IL-2 [118]. Similar to IL-2, it promotes the survival, proliferation and cytotoxic activity of NK cells. IL-12 is another cytokine known to activate NK cells, specifically by stimulating its production of INF- ɣ [119]. Moreover, it has anti-antiangiogenic

effects specifically modulated through NK cells [120]. However, its capacity to induce NK proliferation is lower than that of IL-2 or IL-15 [121]. IL-21 can increase NK cells cytokine productions and cytotoxicity, but in some cases has been shown to limit their proliferation [17]. IL-15 and IL-21 have the added benefits of being able to downregulate regulatory T cell numbers [122, 123].

To our knowledge, aerosolized forms of these cytokines have not been developed. Our lab studied the aerosol gene therapy with PEI: IL-12 in order to eradicate osteosarcoma lung metastasis, but the aerosol delivery of the actual cytokine was not evaluated [124]. In theory, if aerosolized forms of these cytokines were to be developed, we could study their effect on NK recruitment to the lung and its tumoricidal properties. *In vivo* experiments would be designed to compare their capacities to increase injected NK cells in the lung, to increase NK infiltration of osteosarcoma lung metastasis and their overall ability to enhance NK cell therapy. In addition, different combinations of these aerosolized would be evaluated, as studies have shown that they have synergistic effects on NK activation [118, 125]. The combination of IL-2 and IL-21 is of particular interest, as IL-21 addition to IL-2 has the capacity to counteract IL-2's driven regulatory T cell expansion [123].

Exploring the possible effect of aerosol IL-2 on the stimulation of regulatory T cells

The inadvertent stimulation of regulatory T cells is of concern for any therapy involving the use of interleukin-2. Patients receiving IL-2 therapy have

elevated levels of peripheral regulatory T cells [126]. Regulatory T cells can specifically inhibit NK cell activity [83, 127]. Low dose IL-2 therapy increases the proliferation of regulatory T cells in patients with chronic graft-versus-host disease, with no effect on other CD4+ T cells [128]. This may prove a concern since our data shows that treatment with aerosol IL-2 for 5 weeks increases serum IL-2 levels, but to very low levels (Figure 23). Whether or not these levels would correspond to those that selectively increase regulatory T cells would have to be determined. Since we used nude mice for our treatment experiments, any negative effect on therapy that might have been caused by an IL-2 induced increase in regulatory T cells would not have been apparent.

In order to determine if aerosol IL-2 therapy can increase regulatory T cell populations, an animal experiment in which immunocompetent mice are given aerosol IL-2 for at least five weeks would be designed. Treatment for 5 weeks is essential, since at that time we saw increased serum IL-2 levels. The peripheral and lung population of regulatory T cells would be monitored from the day before treatment is started, during the treatment window and afterward. IL-2 serum levels would also be measured through the study. We would compare the regulatory T cell population of these mice before the start of treatment and right after the start of treatment. If there is an increase, we would want to correlate these numbers with the corresponding values of serum IL-2. We would keep monitoring regulatory T cell levels after treatment has ended until serum IL-2 levels have returned to normal. We would also want to monitor CD4+T cell levels

throughout the study, as any increase in regulatory T cell levels might just be proportional to increases in helper T cell levels.

If we do find an increase in regulatory T cell levels caused by low level increases of serum IL-2, we would determine if these correlate with decreased effectiveness of treatment. We would not be able to treat immunocopentnet mice with *ex vivo* expanded human NK cells, so the treatment would have to be limited to aerosol IL-2. We would design a lung metastasis model using BALB/C mice engrafted with mouse metastatic osteosarcoma cell line K7M3. We would treat these mice with aerosol PBS or aerosol IL-2 for 5 weeks. We would sacrifice these mice and extract their lungs at different time points during treatment. We would measure serum IL-2 levels and regulatory T cell populations at the moment of euthanasia. Lung tumors would be stained for TUNEL, mouse NK cell markers and regulatory T cell markers. We would then determine if NK tumor infiltration and tumor apoptosis decreases with increasing serum IL-2 levels and regulatory T cell numbers.

Exploring the effect of high dose and low dose IL-2 in the stimulation of NK cells and its relationship to regulatory T cells

As mentioned above, low-dose IL-2 therapy increases peripheral regulatory T cells [126] . As figure 23 demonstrates, aerosol IL-2 treatment for 5 weeks can cause a very low up regulation of serum IL-2. In the setting of immunocompetent organisms, an upregulation of regulatory T cells could potentially interfere with

NK cell therapy. To address this concern, we could potentially study the effect of high and low doses of IL-2 on NK cell cytotoxicity and proliferation, on regulatory t cells as well as the interactions between the two.

First, we would treat freshly isolated human NK *in vitro* with IL-2 doses that would correspond to low-dose IL-2 therapy that induces expansion of peripheral regulatory T cells *in vivo*. We would measure rate of proliferation, the expression of activating receptors such as NKG2D, and their *in vitro* cytotoxicity against osteosarcoma cell lines. These values would be compared to those from unstimulated NK cells and from NK cells treated with our standard dose of IL-2. Second, we would treated freshly isolated regulatory T cells with the same doses and compare their rate of proliferation and their expression of defining T cell markers. We would be particularly interested in determining if low-dose IL-2 stimulates regulatory T cell proliferation at higher levels than high-dose IL-2 treatment. Subsequently, we would determine the effect of low-dose stimulated regulatory T cell as well high-dose stimulated regulatory T cells on NK cytotoxicity by co-culturing them with NK cells that had been untreated or treated with low-dose or high-dose IL-2.

Chapter 10

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 in complete Dulbecco's Modified Eagle's Medium (Whittaker Bioproducts Inc., Walkersville, MD) supplemented with 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1 mmol/L nonessential amino acids, 2 mmol/L minimal essential medium vitamin solution and 10% heat-inactivated bovine serum (Intergen, Purchase, NJ). LM7 is a human osteosarcoma lung metastatic cell line that was created in our laboratory by repeated intravenous recycling of its parent cell line SAOS-2, through nude mice lungs [64]. CCH-OS-D cells were kindly provided to us by Dennis Hughes, MD, PhD (Division of Pediatrics, The University of Texas MD Anderson Cancer Center). K7M3 mouse osteosarcoma cell line was created in our laboratory by injecting K7M2 intravenously into BALB/C mice and harvesting the lung metastasis [97]. All cell lines were mycoplasma-negative and validated by short tandem repeat DNA Fingerprinting using the AmpFLSTR Identifier kit (Applied Biosystems, Carlsbad, CA). 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

Blood samples were obtained from healthy adult volunteer donors (Gulf Coast Regional Blood Center, Houston, TX) following informed consent. Human NK cells were harvested from buffy coat fractions using the RosetteSep Human NK Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC) and

Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK) [39]. NK cells were cultured in RPMI medium (Cellgro/Mediatech, Manassas, VA) supplemented with 10% heat-inactivated bovine serum (Intergen), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate and 50 IU/ml recombinant human IL-2 (Proleukin, Novartis, Inc., Basel, Switzerland). Genetically-engineered K562 cells with membrane-bound IL-15 and membrane-bound IL-21 were used as artificial antigen presenting cells [aAPC] after 100-Gy g-irradiation for *in vitro* expansion of isolated human NK Cells [39]. Every 3 days cultures were refreshed with media changes. Every 7 days the cultures were re-stimulated with K562s at 1:2 PBMC to aAPC ratio. When further NK cell purity or T cell depletion was required, third party red blood cells were added to enhance agglutination and to remove unwanted cells [129].

Flow Cytometry

The phenotypes of expanded NK cells were analyzed weekly using 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, San Diego, CA) were used to monitor NK cell phenotype. For direct surface staining, NK cells were suspended in FACS buffer (PBS, containing 2% fetal calf serum and 0.1% sodium azide) and incubated with the indicated antibodies for 20 minutes at 4°C. Data was acquired using a FACSCalibur cytometer (BD Biosciences, San Jose, CA) and analyzed using the FlowJo

software (Tree Star, Inc., Ashland, OR). APC and PE-conjugated isotype-control IgG antibodies were used as negative controls (BD Pharmingen). Human NK Cells were defined as $CD56^+$, $CD16^+$, $NKG2D^+$ and $CD3^-$. A purity of at least 95% was deemed appropriate for further use.

Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human HLA-ABC (BD Pharmingen), PE-conjugated mouse anti-human MIC A/B (BD Pharmingen), PE-conjugated mouse anti-human ULBP2/5/6 (R&D Systems, Minneapolis, MN), PE-conjugated mouse anti-human ULBP3 (R&D Systems), and (PE)-conjugated mouse anti-human ULBP1 (R&D Systems) were used to determine HLA and NKG2D ligand expression on human osteosarcoma cells. For direct surface staining, osteosarcoma cells were suspended in FACS buffer (PBS, containing 2% fetal calf serum and 0.1% sodium azide) and incubated with the indicated antibodies for 20 minutes at 4°C. Data was acquired using a FACSCalibur cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Inc.). APC, FITC and PE-conjugated isotype-control IgG antibodies were used as negative controls (BD Pharmingen).

To determine the percentage of proliferating human NK cells, single-cell suspensions derived from *nu/nu* mice lungs, liver, spleen and bone marrow were stained with PE-conjugated mouse anti-human NKp46 (BD Pharmingen) and FITC-conjugated mouse anit-BrdU (eBiosciences, San Diego, CA). For cell surface staining, cells were suspended in FACS buffer (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 in 70% ethanol for 30

minutes on ice, followed by Brdu intracellular staining in FACS buffer (PBS, containing 2% fetal calf serum and 0.1% sodium azide) with anti-Brdu incubation for 20 minutes at 4°C. Data was acquired and analyzed as described above.

Cytotoxicity Assays

NK mediated-cytotoxicity against osteosarcoma cells following a four hour co-incubation period was measured using a [^{3}H]thymidine incorporation assay [130]. Radioactive incorporation was quantified using the Beckman Coulter® LS 6500 Multipurpose Scintillation Counter (Brea, California). The percentage of cytotoxicity was calculated by the following formula: % of cytotoxicity = [(A−B)/A] × 100. A is the radioactivity in counts per minute [cpm] of osteosarcoma cells treated with medium alone, and B is the radioactivity cpm osteosarcoma cells treated with human NK cells.

To determine the importance of NKG2D-ligand interaction, cytotoxicity assays were performed where NKG2D or a NKG2D ligand was blocked. Osteosarcoma cells were plated in triplicate on 96-well plates labeled with 0.2 μCi/well of [^{3}H] thymidine for 24 hours at 37°C, washed twice with PBS and then incubated with 0 μg/mL, 5 μg/mL, or 10 μg/mL mouse anti-human ULBP2/5/6 (R&D Systems) for 24 hours at 37°C. The cells were then washed twice with PBS and co-cultured with increasing concentrations of human NK cells for 4 hours at 37°C, incubated with 0μg/ml, 5μg/ml, or 10μg/ml mouse anti-human NKG2D (R&D Systems). Prior to co-culture with osteosarcoma cells, NK cells were incubated with mouse anti-human NKG2D (R&D Systems) for 1 hour at 37°C.

[^{3}H] thymidine radioactive incorporation and cytotoxicity were quantified as described above.

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. (*Originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc.)*

To evaluate immunohistochemical evidence for NKG2D ligand expression, these microarray tissue sections were deparaffinized in xylene and rehydrated. Incubation at 95°C in citrate buffer for 20 minutes was conducted for antigen retrieval. Incubation with 3% H_2O_2 for 12 minutes was performed to block against endogenous peroxidase followed by protein block with 4% fish gelatin in PBS. Sections were incubated with 50µg/ml recombinant human NKG2D/Fc Chimera (R&D Systems) overnight at 4°C. Horseradish peroxidase goat anti-human secondary antibody (Jackson ImmunoResearch, Inc., West Grove, PA) was then applied for one hour at room temperature [RT] at 1:500 dilution in protein block, followed by 3,3#-diaminobenzidine [DAB] 10 minute incubation and light counterstain with hematoxylin. Sections not exposed to recombinant human NKG2D/Fc Chimera served as negative controls. The expression of the NKG2D ligand ULBP2 is high in terminally differentiated cells in normal human cervix

epithelium [131]. Thus, sections of normal human cervix epithelium (US Biomax Inc., Rockville, MD) were used as positive control.

Animal studies

All animal experiments were approved by the Institutional Animal Care and Use Committee at MD Anderson Cancer Center. Female four-week old athymic *nu/nu* mice and *BALB/C* mice were purchased from the Natural Cancer Institute (Bethesda, MD). All mice were housed in standard cages, at five mice per cage.

Aerosol treatment was performed as described previously [132]. PBS suspension (10 ml) with or without recombinant human IL-2 [IL-2] (TECIN^{TM} Teceleukin, Bulk Ro 23-6019, National Cancer Institute, Frederick, MD) was added to an AeroTech II nebulizer (CIS-USA, Bedford, MA). The nebulizer was operated at a flow rate of 10 liters of air per minute. Mice were placed unrestrained in a sealed plastic cage and exposed to the aerosol for one hour. The aerosol particles were generated with 5% CO_2-enriched air obtained by mixing normal air and CO_2 with a blender (Bird3M, Palm Springs, CA). The CO_2 concentrations were calibrated with a Fluid Fyrite (Bacharach, Inc., Pittsburgh, PA).

To determine human donor NK cell retention in mouse lungs without metastasis, 2 groups of *nu/nu* mice were treated with aerosol IL-2 @ 2000 U or aerosolized PBS. We injected 50 million CM-DiI^{TM} (Molecular Probes, Eugene, OR)-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, Hampton, NH), and frozen. Lungs were expanded with OCT: PBS at a 1:1 dilution before freezing. (*Originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc.)*

In order to compare the proliferation of human donor NK cells in mice, 2 groups of *nu/nu* mice were injected with 50 million human NK cells and treated with either aerosol IL-2 @ 2000 U or aerosolized PBS as described above. Mice were killed 24 and 72 hours after NK cell intravenous injection. They were injected intraperitoneally with BrdU (Invitrogen, Carlsbad, CA) 3 hours prior to euthanasia. The lungs, spleens and livers were harvested and minced. Single-cell suspensions were prepared by passage through cell strainers (BD Biosciences). Bone marrow cells were obtained by flushing femurs and iliac crest with ice cold PBS. Red blood cells were lysed by incubating single cell suspensions in Ammonium Chloride Solution (StemCell Technologies, Vancouver, BC).

In order to assess acute and chronic toxicity of aerosol IL-2, immunocompetent BALB/C mice were treated with either aerosol IL-2 @ 2000 U or aerosol 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 + human NK cells on lung metastasis, 3 million LM7 cells/mouse were injected intravenously through the tail vein of *nu/nu* mice. The presence of micrometastasis was confirmed at 5 weeks in a group of 3 mice. Treatment was then initiated with aerosol PBS, aerosol IL-2, aerosol PBS + human NK cells or aerosol IL-2 + human 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. (*Originally published in Pediatric Blood and Cancer 2013. This material is reproduced with permission of John Wiley & Sons, Inc.)*. Ten animals were used per group. This experiment was repeated twice to verify results. Data shown is representative of one experiment.

To assess aerosol IL-2 toxicity, complete blood count [CBC] and liver enzyme blood chemistry were analyzed in *nu/nu* mice that were 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, Sunnyvale, CA). (*Originally published in Pediatric Blood and Cancer 2013. This*

material is reproduced with permission of John Wiley & Sons, Inc.) Three mice were used per treatment group.

Survival studies were performed using 10 *nu/nu* mice per group using the same method as described above to establish lung metastasis. The presence of micrometastasis was confirmed at 5 weeks in a group of 3 mice. Treatment was then initiated as described above and continued for 5 weeks. Aerosol PBS, aerosol IL-2, aerosol PBS + NK cell and aerosol IL-2 + NK cell treated mice were observed to determine their survival rates.

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-DiI-labeled human NK Cells were used as the controls. (*Originally published in Pediatric Blood and Cancer 2013) This material is reproduced with permission of John Wiley & Sons, Inc.)*

Frozen sections of lung with LM7 osteosarcoma metastasis were also examined for the presence of NK cells using anti-human NKG2D antibody. These frozen sections were incubated with 5% horse serum and 1% goat serum in PBS for 30 minutes and 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 blue (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., Bridgewater, NJ). Mouse NK cells present in LM7 osteosarcoma metastasis were visualized by immunofluorescence using (PE)-conjugated rat anti-mouse NKp46 (BD Pharminogen) as described above.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining.

Frozen tumor sections were fixed with 4% paraformaldehyde and incubated with Proteinase K (Promega DeadEnd Fluorometric TUNEL System, Fitchburg, WI) for 10 minutes, incubated with 3% H_2O_2 for 12 minutes to block endogenous peroxidase, and then incubated over night at 4°C with recombinant terminal transferase (Promega DeadEnd Flourometric TUNEL System) and biotin-16-dUTP (Roche Applied Sciences, Indianapolis, IN). Sections were then treated with 2% bovine serum albumin plus 5% normal horse serum in distilled water for 10 minutes, followed by 30-minute incubation with 4+Streptavidin-HRP-Label (Biocare Medical, Concord, CA). 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 with a Gaussian distribution of values. The Mann-Whitney *U* rank sum was used to evaluate the significance of differences between experimental groups with 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. $P<0.05$ was considered significant. Survival studies were analyzed using the log-rank test.

APPENDIX

Chapter 11

Appendix

Presence of regulatory T cells in the NK cell population

One concern behind any use of IL-2 treatment is the inadvertent activation of regulatory T cells [126], which can limit the immune anti-tumor response. Regulatory T cells are known to suppress the immune response [133, 134], as well as specifically inhibit NK cell activity [83, 135]. Regulatory T cells are defined as $CD4^+CD25^+Fxop3^+$ T cells [136]. Work performed by current lab member Ling Yu, MD (Division of Pediatrics, The University of Texas MD Anderson Cancer Center) demonstrated that aerosol IL-2 @ 2000 U treatment, given every other day for 15 days to immunocompetent BALB/C mice would increase both $CD4^+$ and $CD8^+$ T cells in the lung parenchyma. The number of $CD4^+Foxp3^+$ T cells was also increased, but their increase was proportional to the overall increase in the number of $CD4^+$T cells (unpublished data). Since aerosol IL-2 did not increase the percentage of $CD4^+Foxp3^+$ T cells among $CD4^{+\ T}$ cells, we do not expect the presence of regulatory T cells to hinder the aerosol IL-2 treatment.

Since we used interleukin-2 to expand human NK cells, we wanted to rule out any contamination with and subsequent expansion of regulatory T cells in our *ex-vivo* expanded NK population. We stained for the presence of regulatory T cells in our NK population at different time-points during expansion. Regulatory T cells can be identified as $CD4^+CD25^+CD127^{(low/-)}$[137]. We stained the NK population with Phycoerythrin [PE]-conjugated mouse anti-human CD25 (BD Biosciences), Allophycocyanin [APC]-conjugated mouse anti-human CD4 (BD Pharmingen) and Fluorescein isothiocyanate (FITC)-conjugated mouse anti-

human Cd127(BD Pharminogen). Data was acquired using a FACSCalibur cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Inc.). The percentage of regulatory T cells among our expanded NK population was minimal (Figure A1). Freshly isolated PBMCs from buffy coats contain 0.1 % ± 0.018 regulatory T cells. Expansion for one week significantly increases this percentage to 1.4% ± 0.15 (P = 0.01). This percentage declines to 0.98% ± 0.32 at 2 weeks of expansion and subsequently declines to 0.17% ± 0.12 after 3 weeks of expansion. There was a significant decline in the percentage of regulatory T cells when we compare the population from week 1 and week 2 to the population from week 3 (P = 0.007, P = 0.045; respectively). This demonstrates that, although IL-2 treatment increases the *ex vivo* regulatory T cell population initially, the effect is not sufficient to go in par with NK cell expansion. We believe this low percentage of regulatory T cells will not significantly impair NK cell function. All of our NK cell studies were performed with human NK cells that had been expanded for 3 weeks.

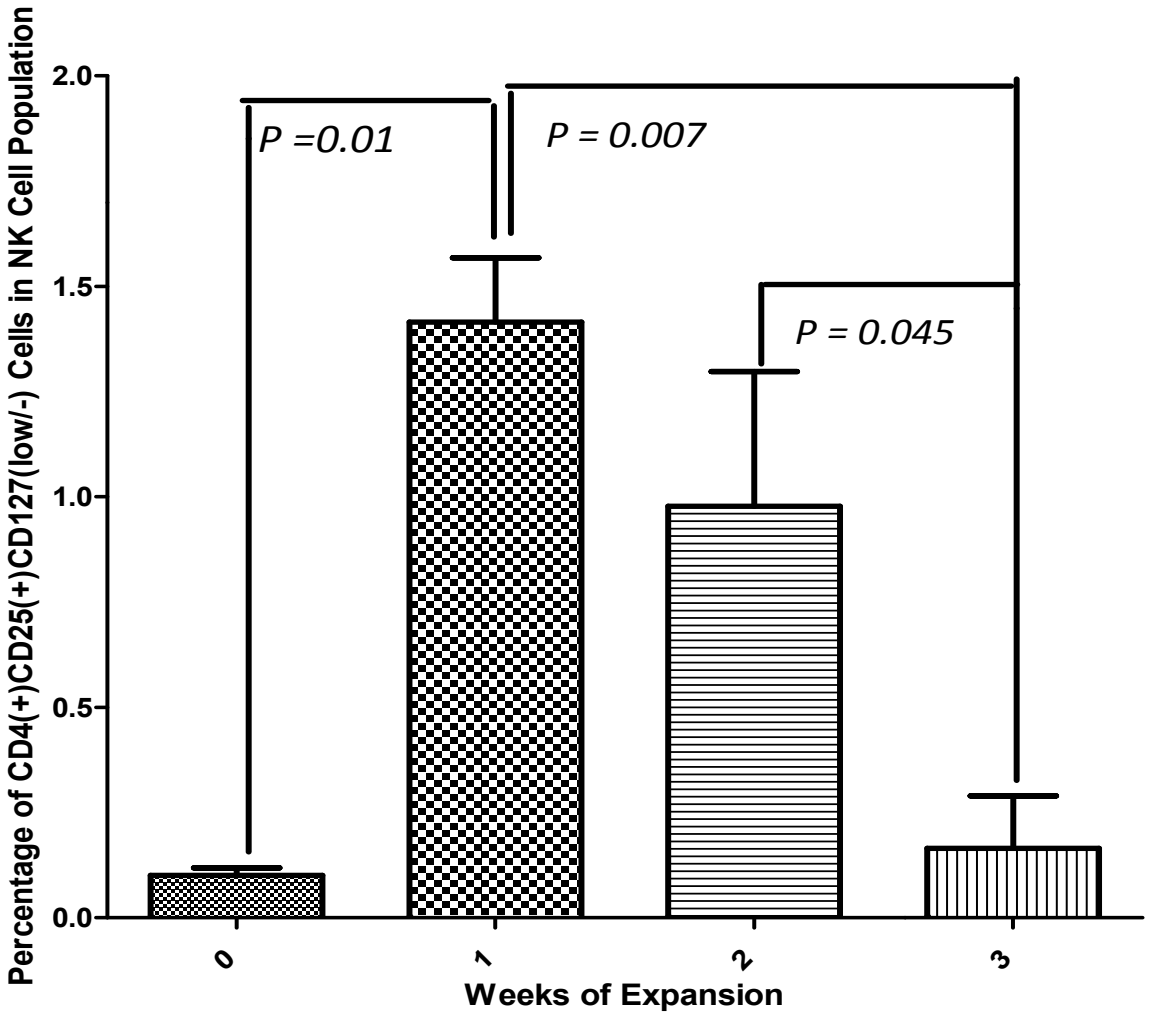


Figure A1: Regulatory T cell population during human NK cell *ex vivo* expansion. *Ex vivo* human NK cell populations were monitored for the presence of regulatory T cells. P<0.5 was considered significant

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