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# EFFECT OF ENTINOSTAT ON NK CELL-MEDIATED CYTOTOXICITY AGAINST OS CELLS AND OS LUNG METASTSIS

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# EFFECT OF ENTINOSTAT ON NK CELL-MEDIATED CYTOTOXICITY AGAINST OS CELLS AND OS LUNG METASTSIS

A

DISSERTATION

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in Partial Fulfillment
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for the Degree of

DOCTOR OF PHILOSOPHY

by

Simin Kiany, MS

Houston, Texas
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## DEDICATION

To my devoted parents and beloved siblings

## ACKNOWLEDGEMENTS

My deepest appreciation goes to my advisor Dr. Eugenie Kleinerman for her support and tireless efforts in bringing out the best in her students. I am sincerely grateful for her guidance and encouragement during my graduate career. She has been my role model both as a great scientist and as a strong woman.

I also express my sincerest gratitude to my advisory and supervisory committee members: Dr. Joya Chandra, Dr. Dean Lee, Dr. Gregory Lizee, and Dr. Kenneth Tsai for their crucial role throughout my journey in graduate school. I appreciate them for their guidance, feedback, and insight.

I would also like to thank the current and past members of Dr. Kleinerman's lab. They have been a family away from home. I am thankful for their help, ideas, and encouragement.

I am so grateful to have a wonderful family: my devoted parents and beloved siblings whose generosity and encouragement throughout the years have been truly inspirational.

EFFECT OF ENTINOSTAT ON NK CELL-MEDIATED CYTOTOXICITY AGAINST OS CELLS AND OS LUNG METASTSIS

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Advisory Professor: Eugenie S. Kleinerman, M.D.

The purpose of this study was to investigate the effect of the HDAC inhibitor entinostat on the efficacy of NK cell therapy for OS lung metastasis. The Lung is the most common site of OS metastatic spread in OS and pulmonary metastasis is the main cause of mortality. We have previously demonstrated that NK cell therapy has minimal efficacy against OS metastasis. We wished to determine whether we could augment the killing of OS cells *in vitro* and improve the efficacy of NK cell therapy *in vivo* by adding oral administration of entinostat. We elected to use our nude mouse human OS lung metastasis model for this purpose. *In vitro*, entinostat increased NK cell ligands on OS cells (MIC A/B, ULBP1, ULBP2/5/6, and CD155) and enhanced the NK cell-mediated cytotoxicity. Entinostat oral administration also increased MICA/B expression on lung tumors. Entinostat (≤ 2 μM) did not have any adverse effect on NK cell viability, receptor expression, or function within the 24 h treatment.

We demonstrated two potential mechanisms by which entinostat enhanced expression of MICA and MICB. Our data showed that entinostat increased the acetylation of histone 4 on the MICA and MICB gene promoters which enhanced MICA and MICB gene transcription. We also showed that entinostat decreased the

expression of mir-20a, mir-93, and mir-106b, microRNAs that up-regulate both MICA and MICB.

Although our findings showed that entinostat augmented NK cell-mediated cytotoxicity against OS cells *in vitro*, the *in vivo* studies failed to show enhanced efficacy of the combination therapy. This may be explained by our finding that while NK cells infiltrated into the lungs and were at the tumor periphery, we were unable to detect the presence of NK cells inside lung tumors. This suggests that adding a cytokine such as IL-2 and IL-21 may enhance the NK cells trafficking into the lung nodules and improve the NK cell therapy efficacy. Entinostat up-regulated the immune inhibitory molecule PD-L1 on OS cells. Therefore, blocking PD/PDL1 interaction by PD-L1 monoclonal antibody may increase the anti-tumor effect of entinostat+ NK cells. Further investigations are necessary to define the specific mechanism of resistance.

## LIST OF TABLES

# Chapter 1

## Introduction: background, rationale and research plan

## Osteosarcoma

Osteosarcoma (OS) is the most common primary bone tumor that originates from transformed mesenchymal stem cells. OS occurs in both children and adults with two peaks of incidence, one at the age of 10-14 years, and the second at age of 65 and older. The survival rate of OS patients is inversely correlated with their age and older patients have a worse prognosis (1). Based on the National Cancer Institute SEER report, the incidence of OS is more frequent in the African American and Hispanic population than in the white population. Although OS can be diagnosed in any bone, the most common sites of occurrence are the femur (42%), the tibia (19%), and the humerus (10%) bones. OS usually initiates in the medullary cavity of long bones and then spreads to the cortex. At the time of diagnosis, 80% of the cases are localized in one bone and about 20% of newly diagnosed patients present with the lung metastasis. Lung is the most common site of metastasis and the main cause of mortality. Bone is known to be the second most common site for metastasis while metastasis to other organs is very rare (2).

The current treatments for OS consists of neoadjuvant chemotherapy followed by surgical resection of tumor and then adjuvant chemotherapy post-operatively. The advantage of neoadjuvant chemotherapy is tumor burden reduction, and elimination of lung micrometastasis. The standard combinational chemotherapy for OS include treatment with cisplatin, doxorubicin, high-dose methotrexate with leukovorin rescue, and ifosfamide. Although there has been remarkable improvement in the prognosis of patients with primary OS over the past 30 years, patients with local relapse or those that develop metastasis following

therapy experience significantly lower survival rates. Current treatments have enhanced the 5-year survival rate of patients with non-metastatic OS to 70%; however, for those who present with pulmonary metastasis, the 5-year survival rate is less than 20% (3-5). New therapeutic modalities are needed to combat OS especially for the disease at the metastatic stage which is resistant to standard treatments such as chemotherapy and radiation.

**Immunotherapy in osteosarcoma**

Poor survival rates in patients with OS pulmonary metastasis highlights the necessity for development of new therapeutic approaches, including immunotherapies. The immune system has a critical role in eliminating cancer, thus any approach to up-regulate immunity against cancer may have a significant impact on OS treatment (6). Current immunotherapy treatments available for cancer can be classified into 6 broad categories:

1- Adoptive immunotherapy:

Adoptive immunotherapy refers to infusion of allogeneic or autologous immune cells (T- or NK-cell) or antibodies in order to boost anticancer immune response (7). Chimeric antigen receptor (CAR) T‑cells that have genetically modified T cell receptors are considered as a promising cellular immunotherapy for cancer. In brief patients T cells are removed and genetically modified with the variable regions of antibodies specific to the targeted antigen on tumor cells and re-introduced into the patient. Thus these specifically re-directed T cells will kill antigen bearing tumor targets (8). Several clinical trials targeting sarcoma are

underway. Two phase I trials targeting the tumor antigen NY-ESO-1 on metastatic and recurrent synovial sarcoma in children and adults (NCT01343043), or on advanced NY-ESO-1-expressing sarcomas in patient receiving radiation (NCT02319824) with CAR T-cells therapy are underway.

It has been shown that IL-11Rα is overexpressed on OS and *in vivo* studies showed that IL-11Rα-CAR T cell therapy resulted in regression of OS pulmonary metastasis (9). Human epidermal growth factor receptor 2 (HER2)-specific CAR T cells resulted in tumor reduction in a mice model established with OS lung metastasis (10). Adoptive immunotherapy using NK cells is considered as another emerging immunotherapeutic modality for cancer which is a major focus of this dissertation.

2. Therapeutic vaccines:

Therapeutic cancer vaccines trigger immune system to attack cancer cells expressing tumor associated antigens. Vigil formerly known as FANG vaccine, which expresses targeted antigen and GM-CSF, for patients with metastatic Ewing sarcoma (NCT02511132) is in a phase II clinical trial; CMB305 vaccine is developed for NY-ESO-$1^{+}$ tumors for patients with advanced disease is in a phase I trial (NCT02387125); DSP-7888 vaccine targeting $WT1^{+}$ cancers including sarcoma (NCT02498665), and dendritic cell vaccine for adults and children with sarcoma (NCT01803152A) are in phase I trials.

3. Checkpoint inhibitors/immune modulators

Checkpoint inhibitors are drugs or monoclonal antibodies used to block the inhibitory receptor/ ligand to enhance the immune response otherwise they are exhausted or energetic, e.g. blocking immune inhibitory molecules PD-1, PD-L1, and CTLA4. Several antibodies blocking these molecules (check-point inhibitors) are under evaluation in clinical trials (11).

Immunomodulation refers to taking advantage of immunomodulatory agents such as cytokines to augment immunity against tumor cells nonspecifically by activating innate immunity (mostly NK cells, monocytes, and macrophages). An example of a promising immunomodulatory agent for OS treatment is Liposome-encapsulated Muramyl Tripeptide-Phosphatidyl Ethanolamine (L-MTP-PE). The mechanism underlying the use of L-MTP-PE is that monocytes and macrophages uptake the liposome-encapsulated MTP-PE and become activated and release pro-inflammatory cytokines e.g. IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) in the tumor microenvironment (12, 13). In relapsed OS patients, L-MTP-PE alone or in combination with ifosfamide significantly increased the disease-free survival rate (14). Adding L-MTP-PE to the standard chemotherapy resulted in a 29% decreased mortality rate in newly diagnosed OS (15).

Interleukin-12 (IL-12), the key inducer of cell-mediated immunity, has also been studied as an immuno-stimulatory agent for OS. Aerosolized delivery of polyethylenimine (PEI) carrying the murine IL-12 gene (PEI: IL-12) to the lung of mice with established OS lung micro metastasis demonstrated therapeutic potential and dramatically reduced the number of lung metastases (16, 17).

Aerosolized PEI: IL-12 also significantly increased the therapeutic effect of ifosfamide for OS pulmonary metastases (18).

4. Oncolytic virus therapy

Oncolytic viruses selectively kill tumor cells by replicating inside the cell whereas normal cells are resistant to this mechanism. A phase I/II trial of Pexa-Vec (JX-594), a virus engineered to lyse cancer cells and induce GM-CSF-driven tumor immunity, for patients with soft tissue sarcoma (NCT02630368) is ongoing.

5. Adjuvant immunotherapies

Adjuvants are substances that are used to boost immune response either alone or in combination with other immunotherapies. Adjuvants can be either stimulating or blocking antagonist ligands. For instance, Poly-ICLC (Hiltonol), a Toll-like receptor 3 agonists is used as an adjuvant for several cancers including sarcoma (NCT02423863)

6. Monoclonal antibodies

Monoclonal antibody-based treatment of immunotherapy for cancer has been established as one of the most successful therapeutic strategies for both blood malignancies and solid tumors for the past 20 years. Monoclonal antibodies targeting CD56-expressing tumors, including synovial sarcoma, rhabdomyosarcoma (NCT02452554), GD2 antigen on neuroblastoma and recurrent osteosarcoma (NCT02502786, NCT02484443), gpNMB antigen

overexpressed by recurrent osteosarcoma (NCT02487979), RANK ligand, and in children with osteosarcoma (NCT02470091) are in phase II clinical trials.

**Natural killer cell biology and function**

NK cells are a type of lymphocyte that play a major role in the innate immune system against virus-infected and tumor cells (19). NK cell had initially been identified in 1975 due to their killing activity against mouse leukemia cells (20). NK cells develop from common lymphoid progenitor cells (CLPs) in bone marrow; however, the precursors of NK cells are not fully characterized in humans. Developed NK cells are distributed in lymphoid as well as non-lymphoid organs i.e. BM, spleen, peripheral blood, lymph nodes and lung and liver (21). NK cells represent 10– 15% of peripheral lymphocytes and are identified by the basic immunophenotype as CD56+ CD3- cells.

In contrast to T cells, NK cells recognize their target cells without needing antigen-specific receptors. Indeed, they become activated by the signals transduced through a group of activating and inhibitory cell surface receptors. The net balance between activating and inhibitory signals define the NK cell fate. When the activating signals exceed the inhibitory signals, NK cells become activated (22). Among activating receptors, natural killer group 2D (NKG2D) is the most well-characterized and effective receptor. NKG2D interacts with its ligands expressed on target cells, including UL-16-binding proteins (ULBPs) 1–6 and the major histocompatibility complex class I (MHC-I) polypeptide-related sequence (MIC) A

and B and. Some other NK cell activating receptors include the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 and DNAX accessory molecule-1 (DNAM-1). The respective ligands for NCRs are still unknown; however, it has been shown that NK cells recognize their target cells at least in part through these ligands. The ligands for DNAM-1, Nectin-2 (CD112) and poliovirus receptor (PVR, CD155) are vastly expressed on several tumors, including sarcomas. Killer cell immunoglobulin-like receptors (KIRs) and the CD94–NKG2A receptor are known as NK cell inhibitory receptors that bind to the classic and non-classic human leukocyte antigen (HLA) class I molecules (23-26).

NK cells kill tumor cells either by secreting cytotoxic granule or by inducing apoptosis through its death-inducing receptors. To induce its cytotoxic function, NK cell needs to contact its target cell through an immunological synapse in which several receptors, signaling molecules and organelles are involved. This ensures specific targeting of the cytolytic process to a single cell within a tissue without adjacent cells being affected (27). Releasing cytotoxic granules (perforin and granzymes) in the immunological synapse is considered as the most effective and fastest way of NK cell killing function (28). NK cells may also induce apoptosis in tumor cells by death-inducing Fas ligand (CD178), tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL), and TNF. These ligands bind to their receptors (FAS and TRAIL receptor) on tumor cells and induce tumor-cell apoptosis (29). NK cell also secrets various cytokines such as IFN-γ which promotes antiangiogenic factors in tumors, increases NK cell cytotoxicity, and stimulates adaptive anti-cancer T cells (30).

**Natural killer cells and cancer immunotherapy**

Adoptive transfer of activated NK cells in the autologous and allogeneic setting has emerged as an effective immunotherapeutic strategy for cancer (31). Although haploidentical hematopoietic stem cell transplant has been effective for clinical benefit of NK cells, growing evidence suggests NK cell activity against carcinomas and sarcomas (32).

Unlike T cells, NK cells have antigen-independent cytolytic activity against tumor cells, thus allogenic NK cells can be infused with a decreased risk of graft-versus-host disease (GVHD) in the recipient (33). Allogeneic *ex vivo* expanded NK cells from healthy donors are also beneficial for cancer patients with nonfunctional NK cells (34). Moreover, allogenic NK cell therapy can be even more effective when there is a mismatch between KIR on donor NK cells and the KIR-ligand on recipient tumor cells due to the absence of NK cell inhibitory signal (35). In fact, enhanced NK cell tumor cytotoxicity owing to incompatibility between KIR and KIR-ligand was the original rational for the improvement of allogeneic NK cell therapy. Allogenic NK cells can be transferred either in a hematopoietic cell transplantation (HCT) setting or as an adoptive immunotherapy. NK cells can be purified and expanded from healthy donor lymphapheresis products, umbilical cord blood, or embryonic stem cells. Various methods for *in vitro* NK cell expansion have been reported which use a combination of different cytokines and feeder cells. Our method for NK cell expansion includes culturing of NK cells on irradiated K562 cell expressing membrane-bound IL21 (mbIL21) supplemented with interleukin-2 (IL-

2). This ethod results in rapid proliferation of NK cells with 21,000 fold expansion in 21 days (36).

The first clinical trial of immune cell therapy was performed by Dr. Steven Rosenberg and his group in 1980s by using lymphokine-activated killer (LAK) cells and IL-2 in patient with metastatic melanoma (37, 38). Autologous PBMCs were incubated with IL-2 to induce LAK cells. The feasibility and safety of allogenic NK cell therapy was first shown by Miller and his colleagues in patients with poor prognosis acute myeloid leukemia (AML). The haploidentical NK cells therapy followed by subcutaneous IL-2 infusion daily for 2 weeks resulted in complete remission in 5 patients out of 19 (31). Success in allogenic NK cell therapy in patients with AML was the beginning of many clinical trials to investigate the safety and efficacy of this cellular therapy for cancers, most of them in hematologic malignancies (33, 39). However, it has been reported that adoptively transferred allogeneic NK cells may have a therapeutic potential in solid tumors as well (40, 41).

Investigations suggest that NK cells are involved in OS prevention and prognosis. A study of 44 children with OS showed that they had fewer numbers of peripheral NK cells compare to the control group, supporting the role of NK cell in preventing OS (42). OS patients who had early lymphocyte recovery after chemotherapy showed a better prognosis than patients with late lymphocyte recovery (43). Moreover, Buddingh and his collogues reported that unlike patients with other types of cancer, NK cell function was normal in

osteosarcoma patients and was able to lyse tumor cells, suggesting that NK cell therapy may be beneficial for patients with OS (44).

Studies have shown that Osteosarcoma cells are sensitive to NK cell-mediated cytotoxicity. For example Duck Cho et al., reported that human OS cell lines are killed by activated NK cells (32). Furthermore, human OS primary cell lines (either from primary OS or from lung metastasis) were susceptible to activated NK cell killing due to the NKG2D-NKG2DL interaction (45). This study also demonstrated that in a xenograft orthotopic OS model (NSG mouse) NK cell therapy followed by IL-2 administration (10,000 IU IP injection/mouse) for 5 days, resulted in significant primary tumor regression, no lung metastases, and an increased survival rate. In our lab we also have shown that aerosol IL-2 combined with NK cell therapy, significantly augments NK efficacy against OS lung metastasis. Nude mouse model injected with human LM7cells i.v. which resulted in the formation of metastasis in the lung. Although expanded human NK cell could decrease the tumor burden in the lung, adding aerosolized IL-2 to the treatment enhanced NK cell efficacy dramatically. Aerosolized IL-2 increased the number of infused NK cells in the lung and in the metastatic tumor, but not in the other organs. Moreover, NK cell therapy with aerosolized IL-2 improved the overall survival of mice with established OS pulmonary metastasis (46, 47).

A phase I study of NK cell infusion after hematopoietic stem cell transplantation in patients with solid tumors (including OS) is ongoing (NCT01287104). Furthermore, infusion of activated NK cell line NK-92 in patients with advanced OS has been completed; however, the results show that the

treatment was not clinically effective (48). Therefore, new approaches to augment the efficacy of NK cell therapy for cancer treatment are necessary.

**Histone deacetylase (HDAC) inhibitors**

Epigenetic modifications such as DNA methylation and acetylation leads to chromatin remodeling, altered gene expression and cellular phenotype. Genetic defects in proteins regulating epigenetics results in loss or gain function and these epigenetic aberrations leads to onset and progression of human disease (49).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are responsible for histone modifications. HAT stimulates gene transcription by adding acetyl group to histones and unfolding the chromatin which in turn enables access for transcriptional machinery to chromatin. While, HDAC remove the acetyl group from histones and results in condense chromatin and less gene expression. In human 18 HDACs have been identified and classified in four classes. Class I includes HDAC 1, 2, 3, and 8; Class II includes HDAC 4, 5, 6, 7, 9, and 10; Class III includes sirtuins; Class IV includes HDAC 11. Abnormal function of HDACs is often associated with tumorigenesis and poor prognosis in cancer (50). Therefore, HDACs have become promising therapeutic targets in cancer and other immune related diseases (51). Several HDAC inhibitors having various target specificity and pharmacokinetics have been synthesized (52) and many of them are in clinical trials for treatment of malignancies. These inhibitors induce senescence, apoptosis, growth arrest, and differentiation of cancer cells. Studies shows that HDAC inhibitors are selectively cytotoxic to tumor cells while normal cells appear

to be resistant (53). Moreover HDAC inhibitors involve in chromatin remodeling by allowing access for transcriptional machinery to chromatin resulting in controlled gene expression, e.g. NK cell related genes NKG2D ligands, killer cell immunoglobulin-like receptor, and 11 Ly49a (54, 55). HDAC inhibitors belong to 4 structural classes: benzamide, hydroxamic acid, cyclic peptides or short chain fatty acid. Entinostat (MS-275) belongs to the benzamide group and known to be a narrow-spectrum HDACi which affect HDAC class I with almost no effect on HDAC 8. Entinostat is in clinical trial for treatment of both solid and hematological malignancies (56).

Though HDAC inhibitors exhibited anticancer activity accumulating evidence suggests that enhanced and efficient anti-tumor activity is achieved by combination with other drugs or adaptive immunotherapy i.e NK cells. Studies have shown that HDAC inhibitors play roles as immune modulators resulting in enhanced recognition of tumor cells by immune cells (57). Cytotoxicity of natural killer cells correlates with the interaction of their receptors (e.g NKG2D) with the ligands (e.g., MICA and MICB) on target cells. Benzamide containing HDAC inhibitor entinostat enhances NK cell- mediated cytotoxicity against cancer cells by up-regulation of both NKG2D on NK cells and corresponding ligands on tumor (58). Further, HDAC inhibitors, including entinostat, increased the susceptibility of Ewing sarcoma cells for NKG2D-dependent cytotoxicity by NK cells (59). Schmudde et.al showed that the HDAC inhibitors SAHA, sodium butyrate, and entinostat treatment increased the susceptibility of prostate carcinoma and medulloblastoma cell lines for NK killing using the same mechanism (60). These

results are also in accordance with published data concluding that HDAC inhibitors increase the susceptibility of tumor cells to NK cell-mediated cytotoxicity by increasing the expression of MICA/B (61-63). The main focus of this thesis is investigating the role of entinostat (MS-275) in enhancing NK cell-mediated killing by upregulating NKG2D ligands on osteosarcoma tumor model as a combination therapy.

**MicroRNA biogenesis and function**

MicroRNAs (miRNAs) are classified as a large family of small, non-coding, and single-stranded RNAs. They consist of 21 to 25 nucleotides and negatively regulate gene expression at the post transcriptional level. The biogenesis of a miRNA happens by a series of processing events (64). The miRNA formation starts with the genome transcription by RNA polymerase II which results in a long Pri-miRNA precursor (pri-miRNA) generation. Later, the nuclear Drosha5 – DGCR86 complex (RNase III Drosha) cleaves pri-miRNA into a 60–70 nt miRNA precursor (pre-miRNAs) which is then transported to the cytoplasm by the protein exportin-5 (61, 65). In the cytoplasm the pre-miRNA will be processed to a mature miRNA by RNase III Dicer (66, 67). The mature miRNA is then incorporated into the RNA-inducing silencing complex (RISC) and binds to 3’ untranslated region (3’-UTR) of the targeted mRNA which induces gene silencing by inhibiting of mRNA translation or by promoting mRNA degradation (68).

MiRNAs play an important role in the control of diverse biological processes such as cell proliferation, differentiation, and death as well as the development and differentiation of hematopoietic and immune cells (69). In cancer, expression of specific miRNAs has been reported to be abnormal. Increasing evidence shows that miRNAs play a crucial role in cancer initiation and progression, by controlling tumor cell proliferation, differentiation, invasion, metastasis formation, and apoptosis (70-72).

Stern-Ginossar and his colleagues introduced a group of miRNAs (miR-520d, miR-373, miR-372, miR-106b, miR-93, and miR-20a) that suppress MICA and MICB expression by targeting MICA and MICB mRNA 3' UTR sites (73). Knowing that many of these miRNAs are up-regulated in various tumors and play a role in tumorigenesis (72, 74-76), they suggested that the overexpression of relevant miRNAs provide a way for tumor cells to escape from immune cell recognition. Overexpression of these miRNAs suppress MICA and MICB expression in tumor cells and prevent them from being recognized by NK cells, CD8+ T cells and γδ T cells that express NKG2D.

**Aim of the study**

Our previous studies demonstrated that NK cell therapy has minimal efficacy against OS metastasis. Considering the critical need for a new therapeutic approaches for metastatic OS we aim to determine whether combining an HDAC inhibitor entinostat with NK cells would augment the efficacy of NK cell therapy against OS lung metastasis. Effect of entinostat on NK cell function investigated both *in vitro* and in our nude mouse human OS lung metastasis model. We also investigated the mechanism by which entinostat up-regulated NK cell ligands on OS cells. **We hypothesized that combining entinostat with NK cell therapy would augment cytotoxic effect of NK cells against osteosarcoma lung metastasis by increasing the ligands for activating NK cell receptors on OS cells.**

**RESULTS**

## Chapter 2

## Entinostat up-regulates ligands for activating NK cell receptors on osteosarcoma cells and makes them more susceptible to NK cell-mediated cytotoxicity

## RATIONAL

Our research group had previously shown that NK cell therapy in combination with aerosol IL-2, significantly decreased tumor burden of human OS lung metastasis in mice. However, this therapy failed to eliminate tumor completely from the lungs (46, 47). It is well known that the level of NK cell-mediated cytotoxicity against tumor cells is highly dependent on the number of NK cell ligands on their surface and the interaction with the corresponding receptors on NK cells. In order to increase the therapeutic efficacy of NK cell for OS pulmonary metastasis, we decided to enhance NK cell ligand expression on OS cells and for that purpose we investigated combining the HDAC inhibitor entinostat with NK cell therapy. We hypothesized that entinostat would sensitize tumor cells to the cytotoxic effects of NK cells, by increasing cell surface ligand expression specific to the NK cell activating receptors. A similar effect of HDAC inhibitors has been reported in other studies. For example, Schmudde et.al, showed that HDAC inhibitors SAHA, sodium butyrate, and entinostat treatment increased the susceptibility of prostate carcinoma and medulloblastoma cell lines for NK killing using the same mechanism (60). Ewing sarcoma cells treated with HDAC inhibitors including entinostat had enhanced susceptibility NKG2D-dependent cytotoxicity by NK cells (59).

To determine whether entinostat would increase NK cell ligands on OS tumor cells *in vitro*, we treated OS cell lines with entinostat and evaluated surface ligand expression by flow cytometry. We also assessed mRNA and protein levels of NK cell ligands by quantitative real-time PCR and western blot respectively. To

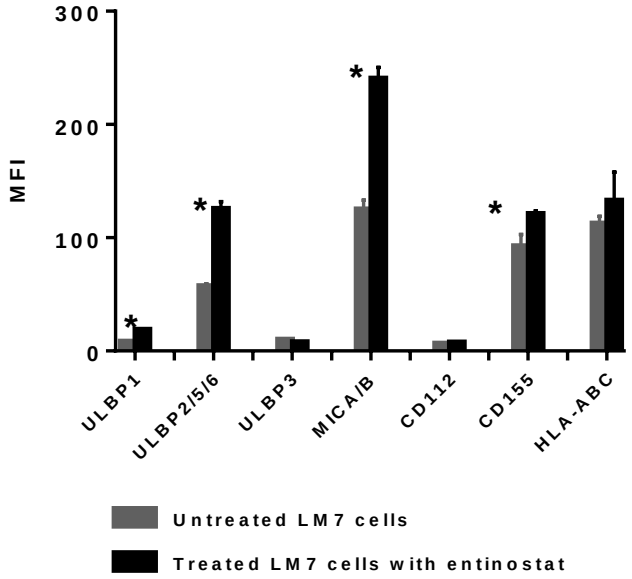
examine whether entinostat enhances OS cell sensitivity to NK cell mediated cytotoxicity, we used the calcein release assay. Using blocking antibodies against NK cell receptors, we showed that NK cell-mediated cytotoxicity against OS cell is dependent on receptor and ligand interaction.

## RESULTS

### Entinostat up-regulates ligands for activating NK cell receptors on OS cells

To determine whether entinostat would increase the expression of ligands specific for activating NK cell receptors on OS cells, various human OS cell lines (LM7, CCH-OS-D, CCH-OS-O, and KRIB) were treated with 2 μM entinostat (≤ IC50) for 48 hours and analyzed by flow cytometry. Entinostat treatment significantly up-regulated ligands for NK cell–activating receptors but did not affect the ligand for the NK cell inhibitory KIR receptor (HLA-ABC). The up-regulated ligands included CD155 (except for CCH-OS-D and KRIB), MIC A/B, ULBP1, and ULBP2/5/6 (figure 1).

A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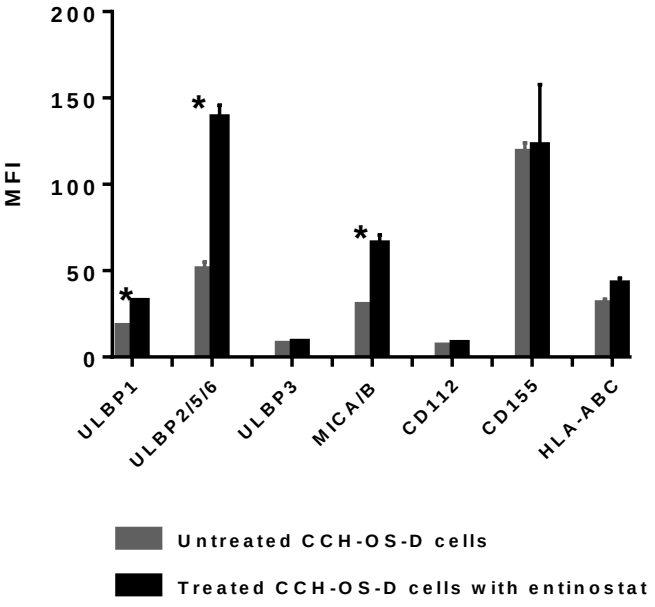
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Treated CCH-OS-D cells with entinostat

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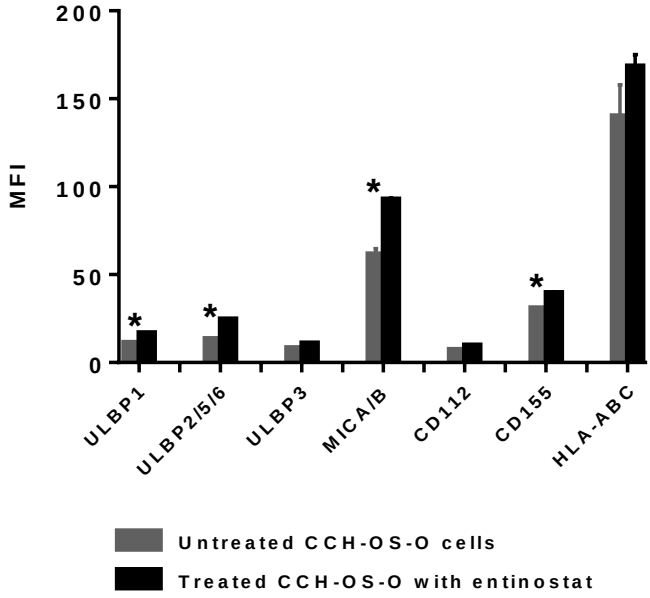
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Treated CCH-OS-O with entinostat

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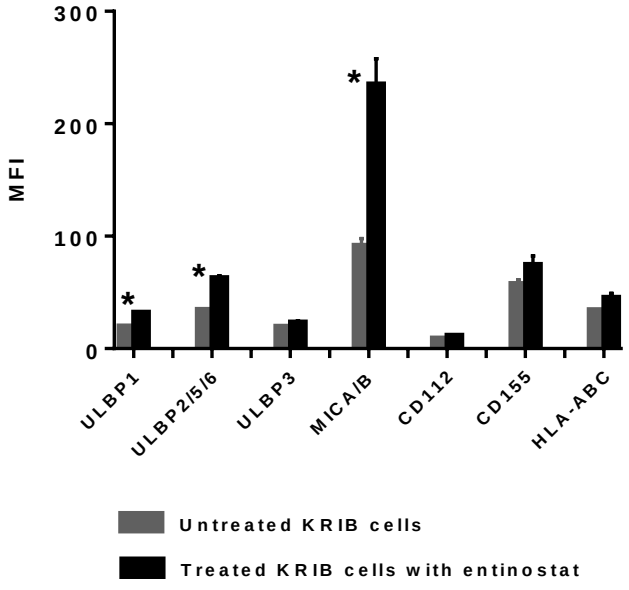
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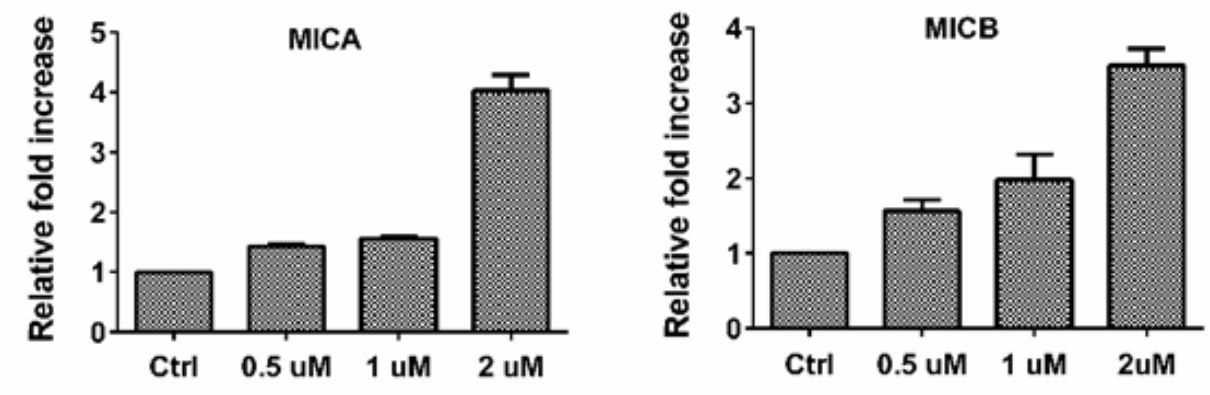
Untreated KRIB cells
Treated KRIB cells with entinostat

**Figure 1. The effect of entinostat on the expression of NK cell ligands on OS cells.** Human OS cells LM7 (A), CCHOSD (B), CCHOSO (C), and KRIB cell (D), were incubated with 2 μM entinostat for 48 hours and NK ligand expression analyzed by flow cytometry. The level of receptor ligand expression is indicated as a mean fluorescence intensity (MFI). P value < 0.05 are marked with *****. All experiments were repeated three times, bars show mean +/- S.E.M.

MICA and MICB are major ligands of NKG2D, and the NKG2D–MICA/B interaction plays a major role in NK cell activation. Therefore, we investigated the effect of entinostat on MICA/B mRNA and protein expression as well. LM7 cells were incubated with 0, 0.5, 1.0, or 2 μM entinostat for 48 hours and total RNA was extracted using Trizol reagent and analyzed by quantitative real-time PCR (qRT-PCR) using primers specific for MICA and MICB. Protein levels of MICA/B from whole cell lysate were analyzed by western blot. LM7 cells treated with entinostat showed increased mRNA (figure 2A) and protein expression (Figure 2B) levels for MICA and MICB in a dose dependent manner.

A



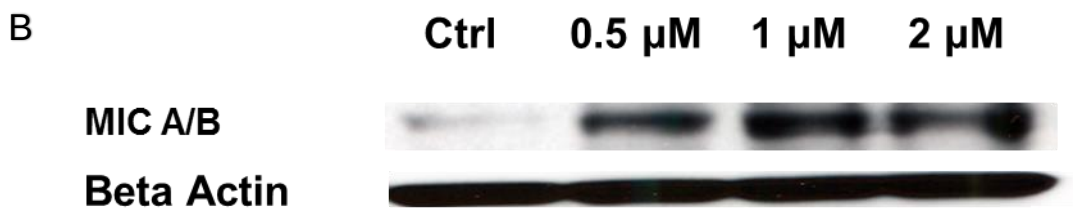



**Figure 2. The effect of entinostat on MICA and MICB mRNA and protein expression in LM7 cells.** (A) LM7 cells were incubated with 0, 0.5, 1.0, or 2 µM entinostat for 48 hours. RNA was extracted and MICA and MICB mRNAs were measured by quantitative real-time PCR using specific primers for MICA and MICB. (B) MICA and MICB protein levels determined by western blot from LM7 whole cell lysate treated with increasing doses of entinostat 0.5, 1.0, or 2 µM. Untreated cells used as control. Bars show mean +/- S.E.M, n=3.

Next, we determined the stable expression of the increased ligands for NK cell receptors on OS cells in response to entinostat treatment. LM7 and CCH-OS-D cells were incubated with 2 µM entinostat, and fresh medium was added after 48 hours. Cells were harvested at the end of 48 h of treatment, at 24, 48, and 72 h after replacing the media. Cells were examined for MICA/B, ULBP1, and ULBP2/5/6 expression by flow cytometry. As mentioned before, treatment with 2 µM entinostat for 48 hours significantly increased MICA/B, ULBP1, and ULBP2/5/6 expression on OS cells (figure 1). The findings from this experiment demonstrated that the expression of up-regulated ligands on OS cells were stable for more than 24 h after the drug was removed from the culture media, suggesting that for the *in vivo* study there can be a window of time between drug administration and the initiation of NK cell therapy since the up-regulated ligands are stable for at least 24 h.

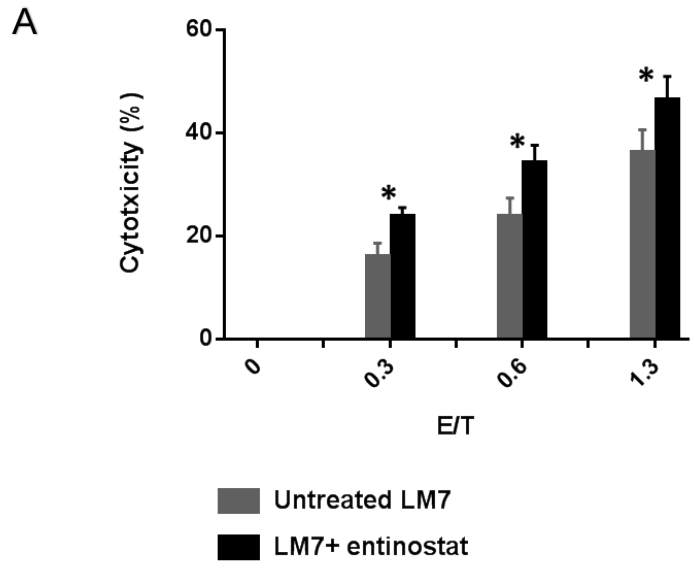
| | ULBP1 | | ULBP2/5/6 | | MIC A/B | |
|---|---|---|---|---|---|---|
| | MFI | | MFI | | MFI | |
| | CCHOSD | LM7 | CCHOSD | LM7 | CCHOSD | LM7 |
| Untreated | 16.3 | 7.9 | 43.0 | 77.0 | 36.9 | 151.0 |
| 2 uM entinostat for 48 h | 29.6 | 29 | 85.8 | 156 | 61.5 | 335 |
| 24 h after drug withdrawal | 30.7 | 24 | 86.0 | 150 | 72.6 | 306 |
| 48 h after drug withdrawal | 26.7 | 16 | 77.7 | 86 | 67.4 | 273 |
| 72 h after drug withdrawal | 27.1 | 11.2 | 74.3 | 67 | 70.3 | 270 |

**Table 1. Up-regulated NK cell ligands on OS cells by entinostat are stable for more than 24 h.** LM7 and CCH-OS-D cells were treated with 2 μM entinostat for 48 hours followed by replacing the conditioned media with fresh media. Cells were harvested at the end of 48 hours treatment and 24, 48, and 72 h after replacing the media and analyzed by flow cytometry with antibodies specific for MICA/B, ULBP1, and ULBP2/5/6.

## Entinostat increases OS cell susceptibility to NK cell-mediated cytotoxicity

Having shown the stable expression of ligands for NK cell receptors on OS cells in response to entinostat treatment, we next hypothesized that the increased expression of ligands on OS cells would enhance NK-cell mediated cytotoxicity. The underlying rationale was that increased expression of NK cell ligands on tumor cells would make them more susceptible to NK cell-mediated cytotoxicity. To prove our hypothesis, control and entinostat-treated LM7 cells were incubated with 1 μM calcein-AM at 37$^{o}$C for 1 hour and then were co-cultured with *ex vivo* activated NK cells in various effector-to-target cell ratios (0.3, 0.6, and 1.3) for 4 hours. The

amount of lysis was quantified using a fluorescence plate reader at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Our findings revealed that OS cells treated with entinostat were more sensitive to NK cells and were killed more effectively than control cells (Figure 3A and 3B).



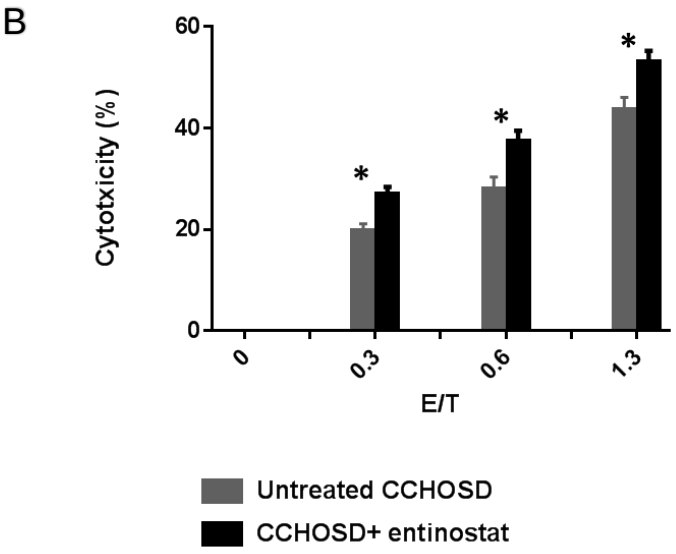
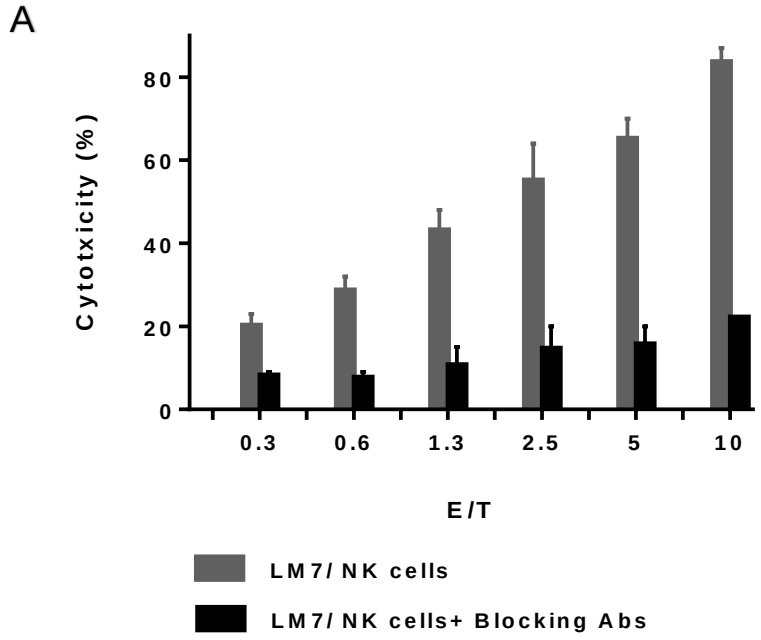



**Figure 3. The effect of entinostat on OS cell susceptibility to NK cell-mediated cytotoxicity.** Entinostat treated and control OS cells (A) LM7 (B) CCHOSD were labeled with 1 µM calcein-AM at 37°C for an hour and washed with RPMI media and co-cultured with *ex vivo* expanded NK cells at effector to target cell (E:T) ratio of 0.3, 0.6, and 1.3 for 4 hours. P value < 0.05. Data pooled from three independent experiments. Bars show mean +/- S.E.M.

To demonstrate that NK cells recognize and kill OS cells via NK cell receptor and ligand interaction, we blocked NK cell receptors with the use of blocking antibodies specific to NKG2D, NKp46, and DNAM receptors and then co-cultured them with untreated LM7 or 2 µM entinostat treated LM7 cells for 48 hours. NK cells not treated with blocking antibodies were used as control. Our data indicated that blocking NK receptors abolished NK cell cytotoxic activity against both untreated and treated OS cells (Figure 4A and 4B, respectively), confirming that NK cell-mediated cytotoxicity for OS cells is dependent on receptor and ligand interactions.



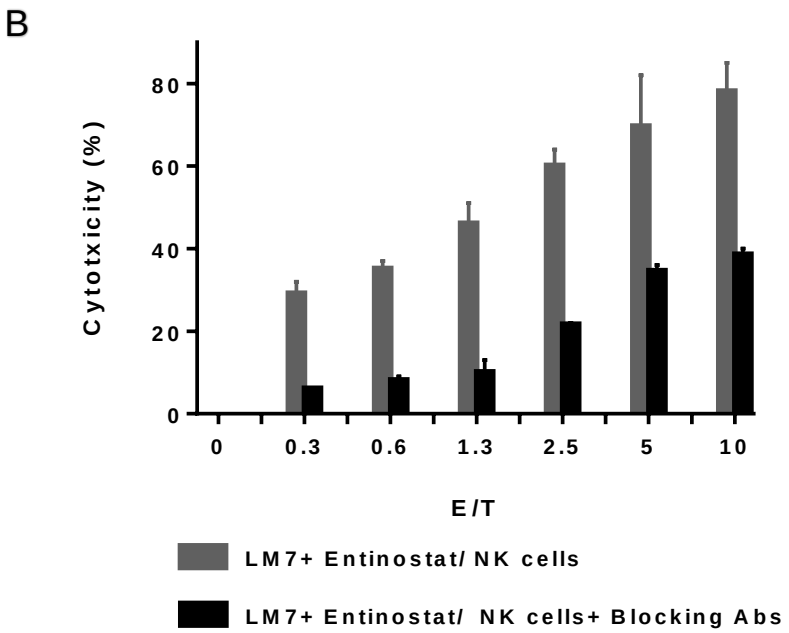



**Figure 4. NK cell-mediated cytotoxicity against OS cells is dependent on receptor and ligand interaction.** Untreated LM7 cells or pretreated LM7 cells with 2 µM entinostat for 48 hours (Fig 4A and 4B, respectively) were used in calcein release assay. LM7 cells were labeled with 1 µM calcein-AM at 37ºC for 1 hour and co-cultured with *ex vivo* expanded NK cells pre-treated with blocking antibodies specific to NK cell receptors for 4 hours at 37ºC. NK cells not treated with blocking antibodies were used as control. Bars show mean +/- S.E.M, n=3.

## SUMMARY

These data support our hypothesis that entinostat enhances the expression of ligands for activating NK receptors on OS cells and sensitizes them for NK cell-mediated cytotoxicity. Entinostat increased MICA/B, ULBP1, and ULBP2/5/6 on four human OS cell lines (LM7, CCH-OS-D, CCH-OS-O, and KRIB), making them more susceptible to NK cell mediated killing. The increased ligands are stable for more than 24 hours after drug removal from the culture media. By blocking NK cell receptors, we also demonstrated that NK cells recognized and lysed OS cells through interaction between NK cell receptors and their corresponding ligands on the OS cells.

# RESULTS

## Chapter 3

## Entinostat does not affect NK cell viability, receptor expression, or cytotoxic function

## RATIONALE

Since the final goal of this study was to use combination therapy with NK cells along with entinostat for the treatment of OS lung metastasis in a mouse model, we needed to determine whether the drug had any adverse effects on NK cell viability, receptor expression, and cytotoxic function to ensure that NK cell therapeutic efficacy in clearing tumors is not compromised. It has been reported that HDAC inhibitors including suberoylanilide hydroxamic acid (SAHA) and valproic acid reduced NK cell cytotoxic function against leukemic cells due to their inhibitory effects on NK cell activating receptor expression (77). However, based on another study, entinostat increased NK cell-mediated cytotoxicity against sarcomas by up-regulating both NK cell activating receptors and their ligands on tumor cells (58). To evaluate the effect of entinostat on NK cells, we treated *ex vivo* expanded and activated NK cells with various doses of the drug for 24 and 48 hours and then examined the NK cell viability, receptor expression and cytotoxic function.

## RESULTS

### Entinostat has no effect on NK cell viability

To determine whether entinostat has any effect on the viability, NK cells were expanded ex *vivo* for 4 weeks and treated with 0, 0.1, 0.5, 1.0, and 2.0 μM entinostat. Cells were collected after 24 and 48 hours and their viability was

assessed using Vi-CELL. Our data indicated that neither 24 nor 48-hour treatment with various doses of entinostat affected NK cell viability (figure 5).

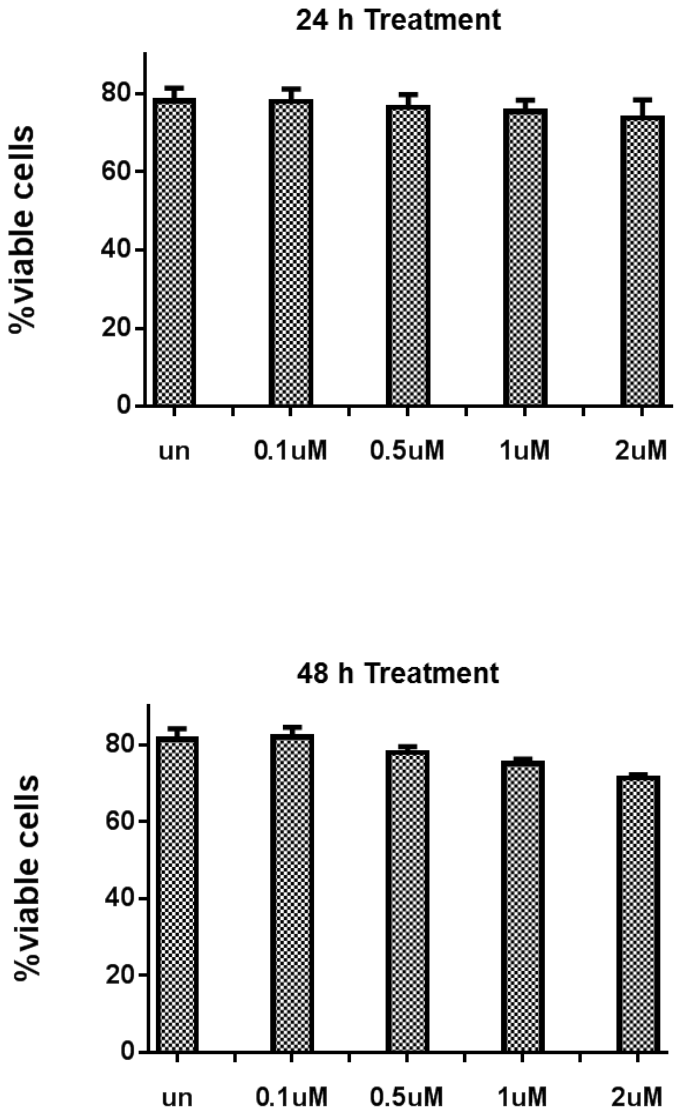
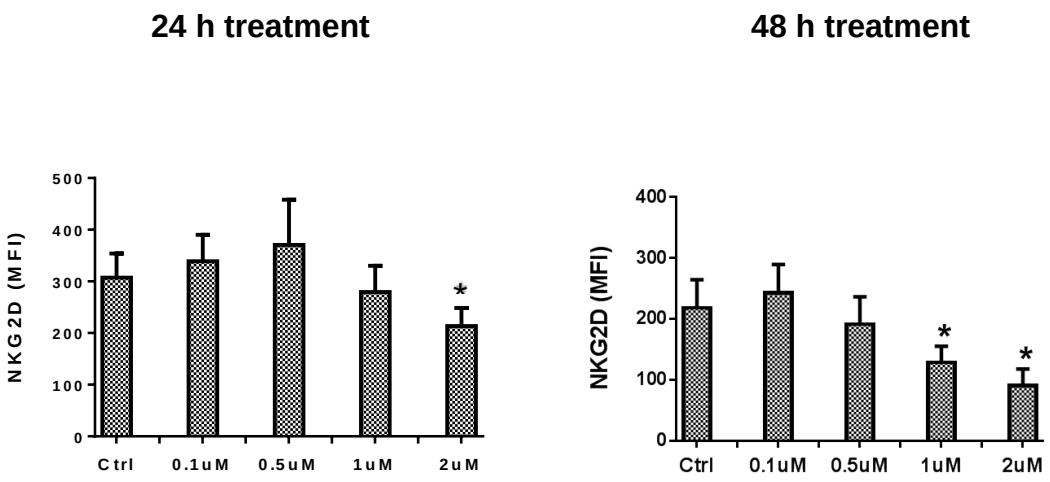



**Figure 5. Entinostat does not affect NK cell viability.** E*x vivo* expanded NK cells were incubated with various concentrations of entinostat 0, 0.1, 0.5, 1.0, and 2 μM. Cells were harvested after 24 and 48 hours and NK cell viability was assessed by using Vi-CELL. Untreated NK cells were used as control. Data was shown as mean +/- S.E.M, n=3.

**NK cell receptor expression is not affected by 24 hour treatment with entinostat**

To determine whether entinostat had any negative affect on NK cell receptor expression, *ex vivo* expanded NK cells were treated with 0, 0.1, 0.5, 1.0, and 2.0 μM entinostat for 24 and 48 hours. NK cell receptors NKG2D, NKp30, NKp44, NKp46, and DNAM-1 expression was analyzed by flow cytometry. Though dose dependent entinostat treatment for 24 hours did not change NK cell receptor expression, after 48 hours higher doses of the drug decreased NK cell receptors expression, except for the DNAM-1 (figure 6). These findings suggests that entinostat concentration of ≥ 0.5 μM for 48 hours may down-regulate the expression of NK cell activating receptors. Thus, for the in *vivo* study, care has to be taken not to administer the drug and NK cells on the same day, but with at least 24 hours duration between drug and NK cell administration to avoid adverse effects on NK cell receptor expression.



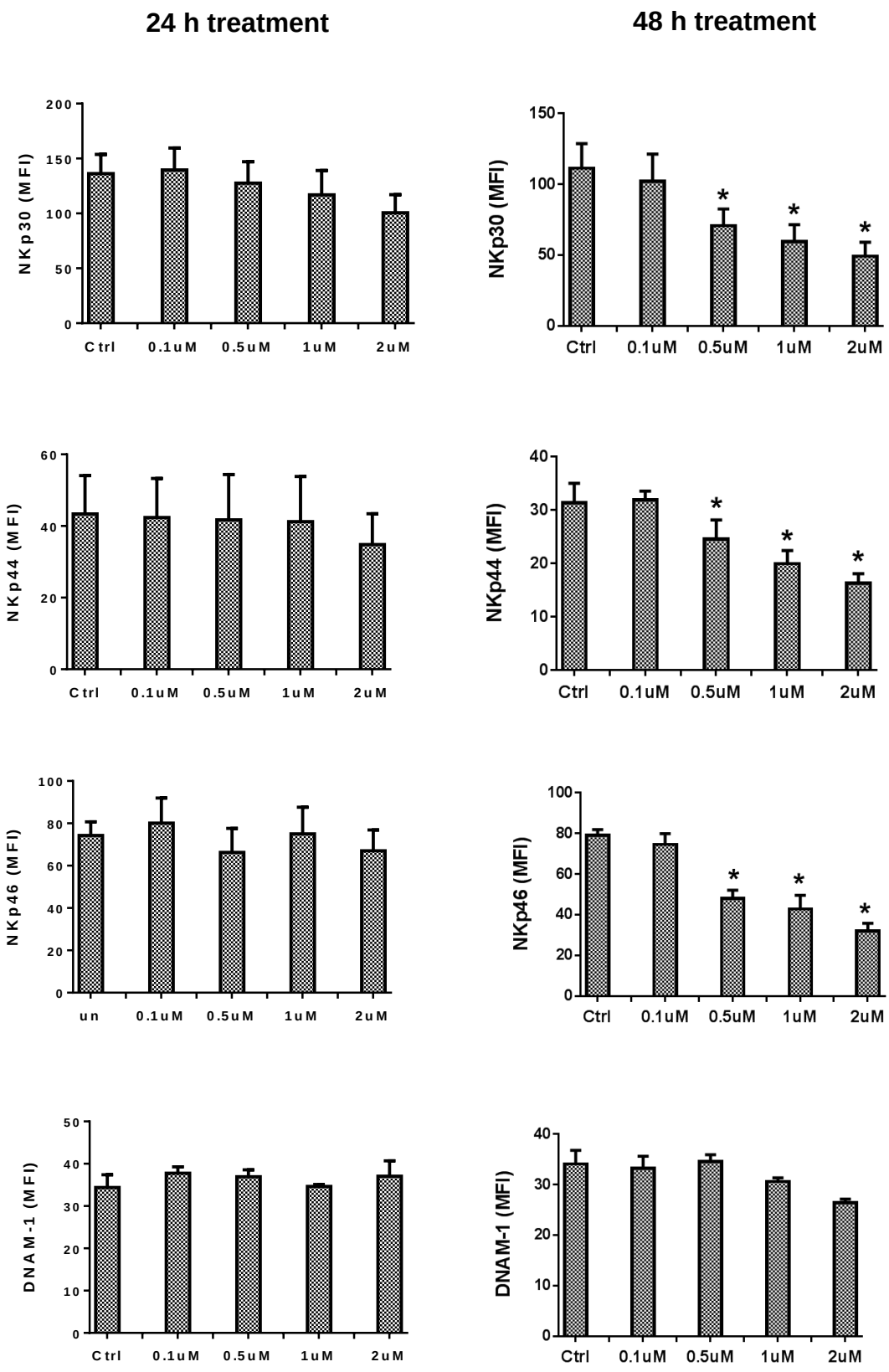

24 h treatment
48 h treatment
NKp30 (MFI)
NKp44 (MFI)
NKp46 (MFI)
DNAM-1 (MFI)
Ctrl
0.1uM
0.5uM
1uM
2uM
un
*

**Figure 6.Treatment with entinostat for 24 hours does not affect NK cell receptor expression.** *Ex vivo* expanded NK cells were treated with 0, 0.1, 0.5, 1.0, and 2.0 µM entinostat for 24 and 48 hours. Cells were stained with antibodies specific to NKG2D, NKp30, NKp44, NKp46, and DMAM-1. NK cell receptors expressions were analyzed by flow cytometry. The expression levels were shown as a mean fluorescence intensity (MFI). P value < 0.05 are marked with *****. Bars show mean +/- S.E.M, n=3.

**Entinostat treatment does not affect the NK cell-mediated cytotoxicity against OS cells**

Having shown that entinostat treatment did not alter NK cell receptor expression within 24 hours of treatment, we also sought to determine whether entinostat had any negative effect on NK cell cytotoxic function. OS cells were incubated with 1 µM calcein-AM at 37ºC for 1 h. Cells were washed with RPMI media and co-cultured with control or entinostat-pretreated NK cells (2 µM for 24 h) at the effector: target cell ratio of 0, 0.3,0.6,1.3, 2.5,5,10. Plates then were incubated at 37ºC for 4 h. Following incubation, 100 µL of the supernatant was harvested and transferred to a new plate and absorbance was assessed using a spectrophotometer. Our results indicated that NK cells pre-treated with entinostat had almost the same level of cytotoxicity against both LM7 and CCH-OS-D cells compared to the cytotoxicity of untreated NK cells (figure 7), suggesting that entinostat does not abrogate NK cell functional activity within 24 hours of treatment.

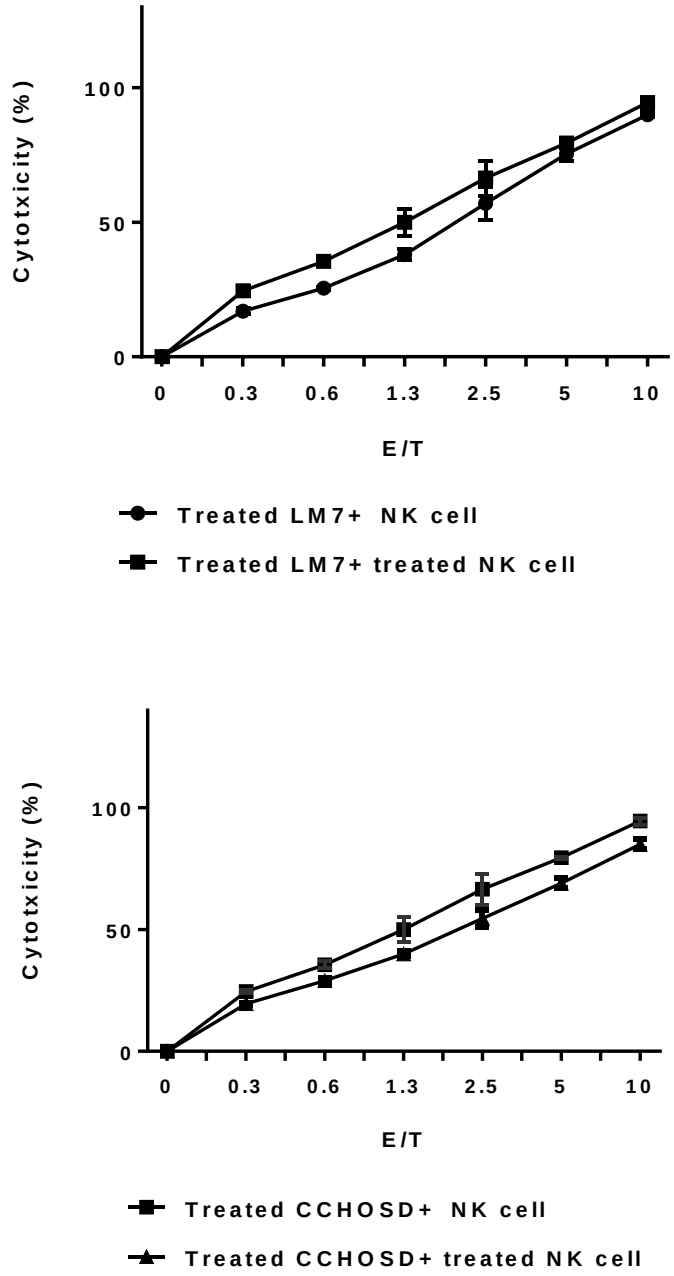



**Figure 7. Entinostat does not reduce NK cell-mediated cytotoxicity against OS cells within 24 h treatment.** OS cells were incubated with 1 µM calcein-AM at 37$^{o}$C for 1 hour. Cells were washed with RPMI media and co-cultured with pretreated NK cells with entinostat (2 µM for 24 h) at a ratio of 0, 0.3,0.6,1.3, 2.5,5,10 (effector cell: target cell). Untreated NK cells served as control. Plates then were incubated at 37$^{o}$C for 4 h. A total of 100 µL of supernatant was transferred a new plate. The plate was read at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Bars show mean +/- S.E.M, n=3.

## SUMMARY

Our data support that 48 h treatment with up to 2 μM entinostat is not cytotoxic for *ex vivo* activated NK cells and does not reduce NK cell viability. Furthermore, the drug does not decrease NK cell activating receptor expression within the 24 h of treatment; however, our finding showed that 48 h treatment with ≥ 0.5 μM may affect the NK cell receptor expression. These data suggest that for in *vivo* studies it is optimal to administer the treatments on different days to avoid any possible adverse effect of the drug on NK cell function. As we have shown, there is stable expression of the up-regulated NK cell *ligands* on OS cells in response to entinostat treatment (Table 1). But the NK cell *receptor* expression is decreased with > 24 h entinostat treatment. Then we decided to have 24 hours in between the entinostat treatment and infusion of NK cells to avoid any adverse effect on NK cell cytotoxicity. Finally, we have shown that 24 h pretreatment with entinostat does not affect the NK cell-mediated cytotoxicity against OS cells (Figure 7).

# RESULTS

## Chapter 4

## Entinostat controls MICA/B expression in OS cells by increasing acetylation of histone 4 linked to the MICA and MICB gene promoters.

## RATIONALE

We demonstrated previously that entinostat increased the expression of ligands for activating NK cell receptors on OS cells (MIC A/B, ULBP1, and ULBP2/5/6). Next we wished to investigate the underlying mechanism by which entinostat enhances expression of these ligands on OS cells. We decided to focus on MICA and MICB, known to be the most important ligands for NKG2D. As an HDAC inhibitor, entinostat stimulates gene transcription by adding acetyl group to histone and unfolding the chromatin which in turn enables access for transcriptional machinery to chromatin (51). We hypothesized that histone acetylation by entinostat at MICA and MICB gene promoters leads to increase in MICA and MICB gene expression in OS cells. A previous study showed that increased acetylation of histones3 (AcH3) binds to the promoters of MICA and MICB is correlated with enhanced MICA and MICB expression in a colon carcinoma cell line treated with entinostat (58). The histone deacetylase inhibitor SAHA has also been shown to enhance MICA/B gene transcription by promoting MICA-associated histone acetylation (78).

To examine our hypothesis we first investigated whether entinostat increases total acetylated H3 and H4 in LM7 cells by using western blotting. Thereafter, chromatin immunoprecipitation (CHIP) assay was performed to compare the level of acetylated histone 3 and histone 4 in untreated and treated LM7 cells (2 μM entinostat for 48 hours).

## RESULTS

### Entinostat increases acetylated H3 and acetylated H4 in OS cells in a dose dependent manner.

We first determined whether acetylation of Histone 3 and 4 is increased in LM7 cells treated with entinostat. Lysates of control and LM7 cells pretreated with 0.5, 1.0, or 2.0 μM entinostat for 48 hours was prepared and acetylated H3 and H4 protein levels were analyzed by western blotting. Our results showed that entinostat enhanced acetylated H3 and acetylated H4 expression in a dose dependent manner compared to untreated control cells (Figure 8).

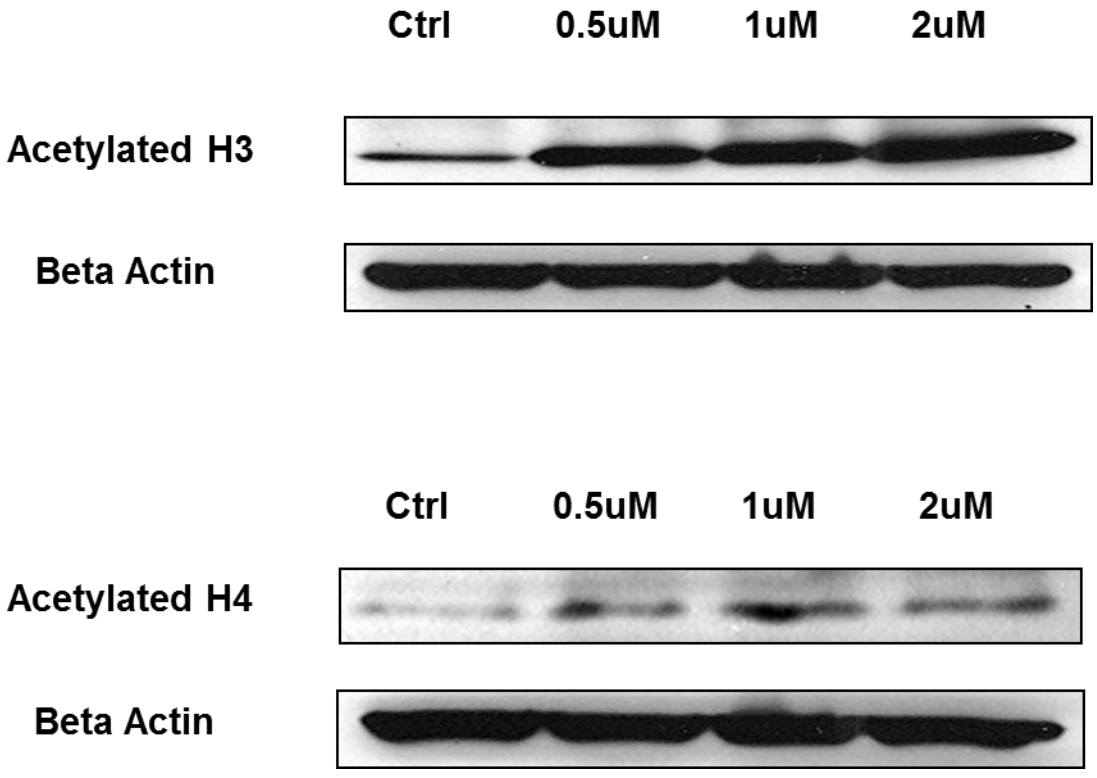


**Figure 8. Entinostat increases acetylated H3 and acetylated H4 in LM7 in a dose dependent manner.** LM7 cells were treated with 0, 0.5, 1.0, or 2.0 μM entinostat for 48 h. Cells were harvested and proteins were extracted. Western blotting was performed using rabbit anti-human acetyl-histone3 or rabbit anti-human acetyl-histone4 antibodies to detect total acetylated H3 and acetylated H4 levels, respectively.

**Acetylation of histone 4 linked to the MICA and MICB gene promoters contributes to up-regulation of MICA/B expression by entinostat.**

We performed the CHIP assay to confirm that one of the mechanism by which entinostat up-regulates MICA and MICB gene expressions in OS cell is by enhancing MICA- and MICB-associated histone acetylation. LM7 cells were treated with 2 μM entinostat for 48 hours and cell lysates were treated with 1% formaldehyde to crosslink protein and DNA. Lysates were sonicated to shear chromatin to 200-1000 bp in length. Then, chromatins were immunoprecipitated by using the following antibodies: anti-acetyl-histone H3, anti-acetyl-histone H4, anti-histone H3, anti-histone H4, or control IgG. MICA and MICB promoter regions were amplified by using quantitative real-time PCR. Two different sets of primers for MICA or MICB promoters and two different antibodies specific for acetylated H3 were used to confirm the accuracy of the results. After normalizing the readings to inputs, the results were presented as a ratio of acetylated histone (H3 or H4) to total histone (H3 or H4). The data suggested that entinostat significantly increased acetylated H4 linked to the MICA and MICB promoters, but did not have any significant effect on acetylation of H3 (Figure 9). Together, these data suggest that increased acetylation of H4 associated with MICA and MICB gene promoters may play a role in enhancing MICA and MICB expression induced by entinostat.

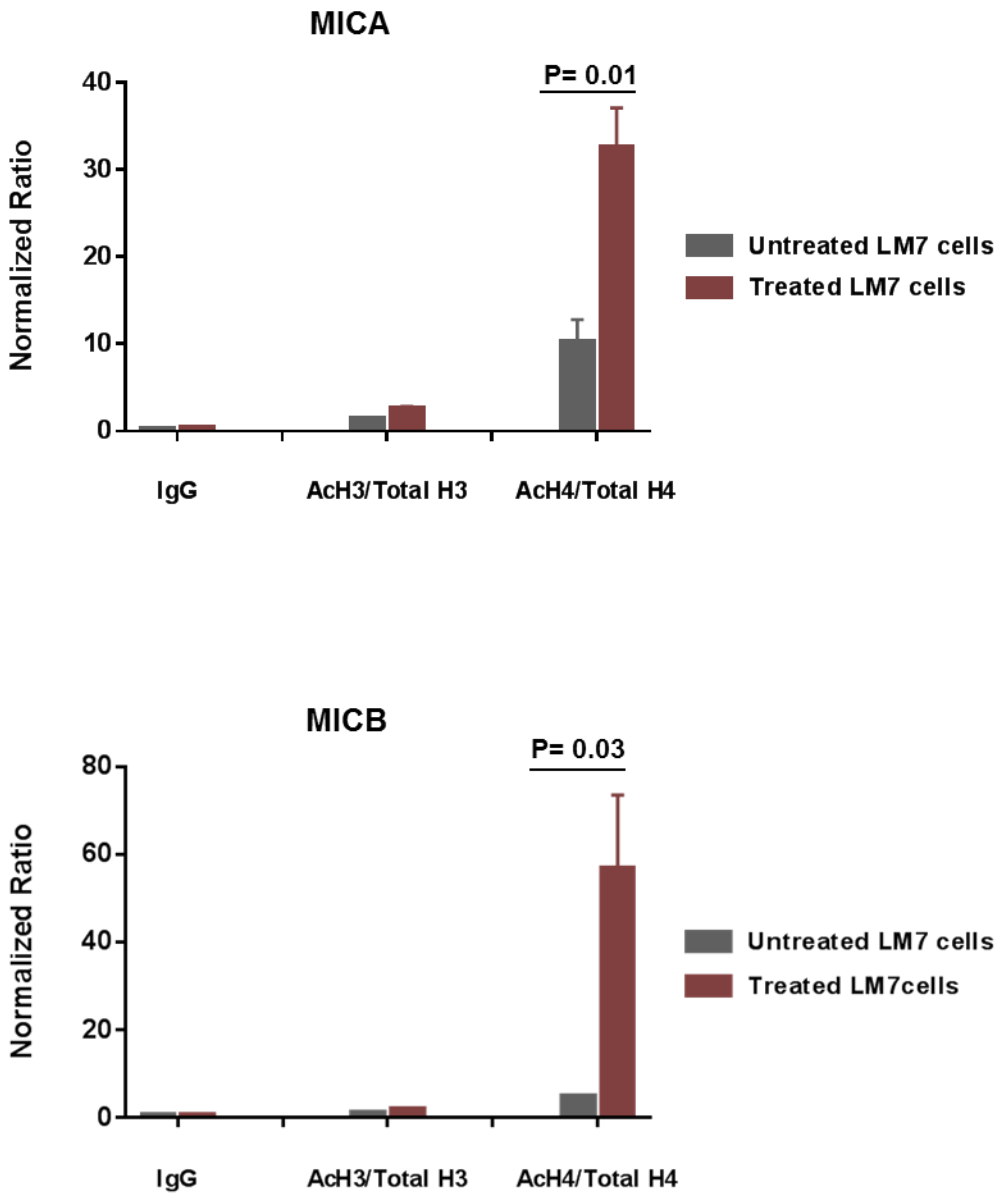



**Figure 9. H4 acetylation associated with MICA and MICB gene promoters contributes to up-regulation of MICA/B expression by entinostat in OS cells.** LM7 cells were treated with 2 µM entinostat for 48 h. Chromatins were immunoprecipitated using the following antibodies: anti-acetyl-histone H4, anti-acetyl-histone H3, anti-histone H4, anti-histone H3, or control IgG. MICA and MICB promoter region were amplified by using quantitative real-time PCR. Two different set of primers for MICA or MICB promoters and two different antibodies against acetylated H3 were used to confirm the accuracy of the results. Bars show mean +/- S.E.M, n=3.

## SUMMARY

We have shown previously that entinostat increases ligands for NK cell receptors on OS cells (figure 1). In this chapter we aimed to explore the underlying mechanism of how entinostat upregulates NK cell ligands on OS cells with the main focus on NKG2D ligands MICA and MICB. We investigated whether up-regulation of MIC A/B expression by entinostat is associated with increased histone acetylation of MICA/B gene promoters, using CHIP assay. Our findings showed that entinostat increased acetylated H3 and acetylated H4 in LM7 in a dose dependent manner. CHIP assay revealed that entinostat enhanced the accumulation of acetylated histone 4, but not acetylated histone 3 on chromatin linked to both MICA and MICB genes.

# Chapter 5

## Entinostat controls MICA and MICB expression in osteosarcoma tumor cells by down-regulating miR-20a, miR-93, and miR-106b expression.

## RATIONALE

Stern-Ginossar et. al., reported that cellular miRNAs including miR-520d, miR-373, miR-372, miR-106b, miR-93, and miR-20a control MICA and MICB expression by targeting their mRNA 3' UTR sites (73). They demonstrated that miR-20a, miR-93, miR-106b were ubiquitously expressed in all the cell lines that they examined (including: 293T, JEG3, DU145, RKO, HFF, and Me1-A1 cells); however, miR-372, miR-373, and miR-520d expression was dependent on cell type. Consistent with their results our findings also confirmed no expression of miR-372, miR-373, and miR-520d in OS cells. Therefore, we chose to study the role of miR-20a, miR-93, and miR-106b in MICA and MICB expression in OS cells.

miRNAs can inhibit gene expression by binding to the 3'UTR of the targeted mRNA resulting in either inhibition of mRNA translation or its degradation. In this chapter we also sought to understand the mechanism by which miR-20a, miR-93, and miR-106b down-regulate MICA and MIC/B expression in OS cells.

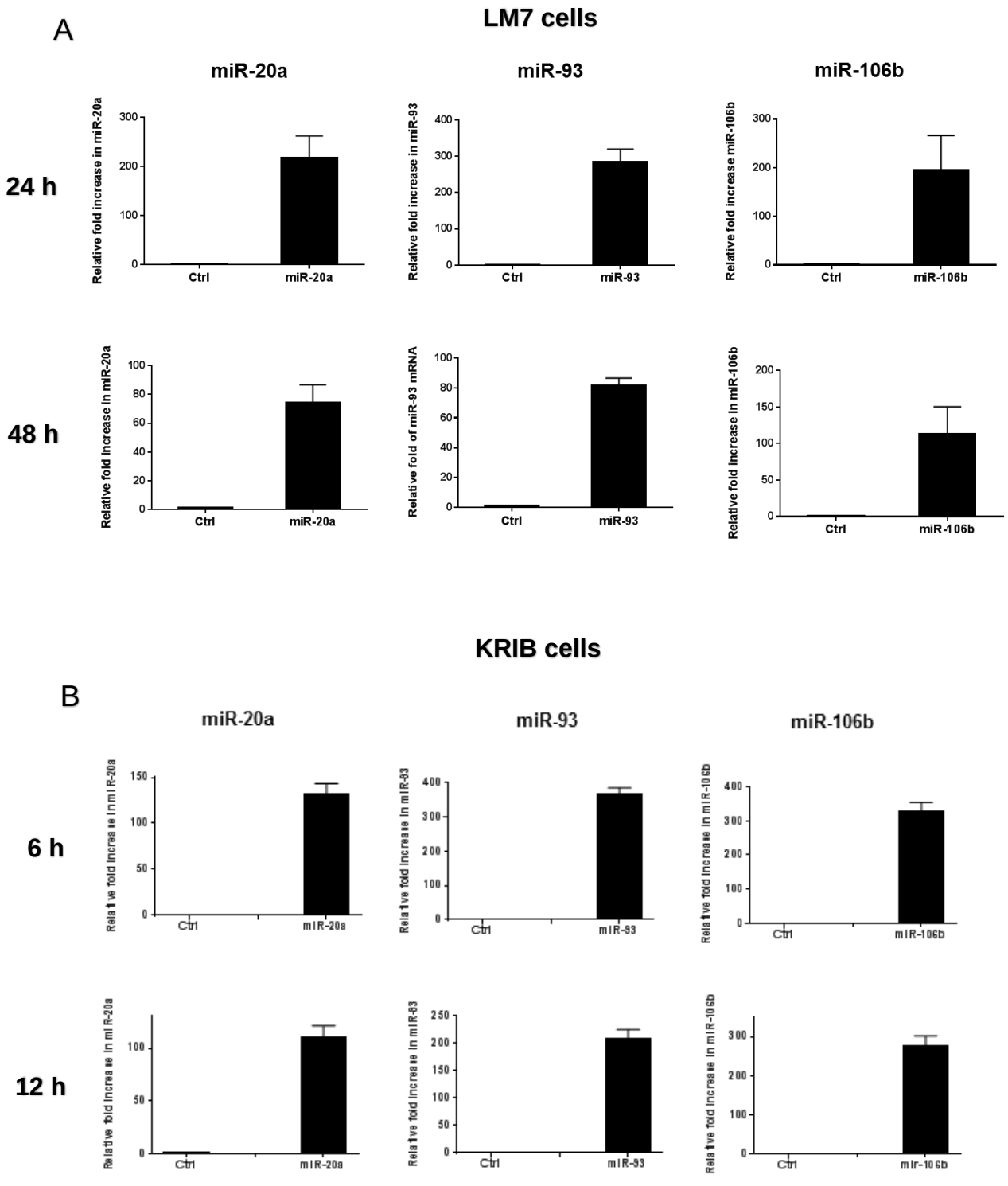
We have shown in chapter 4, figure 9 that increasing the acetylation of histone 4 on chromatin linked to the MICA and MICB gene promoters might be one of the mechanism that entinostat uses to up-regulate MICA and MICB expression on OS cell surface. In this chapter we further demonstrate another molecular mechanism by which entinostat can increase MICA and MICB expression, i.e. through down-regulation of miR-20a, miR-93, and miR-106b expression. Similarly, a study done by H Yang et.al, indicated that the histone deacetylase inhibitor SAHA also up-regulated MICA expression in hepatocellular carcinoma cells (HCC) by suppressing miR-20a, miR-93 and miR-106b expression (78).

## RSULTS

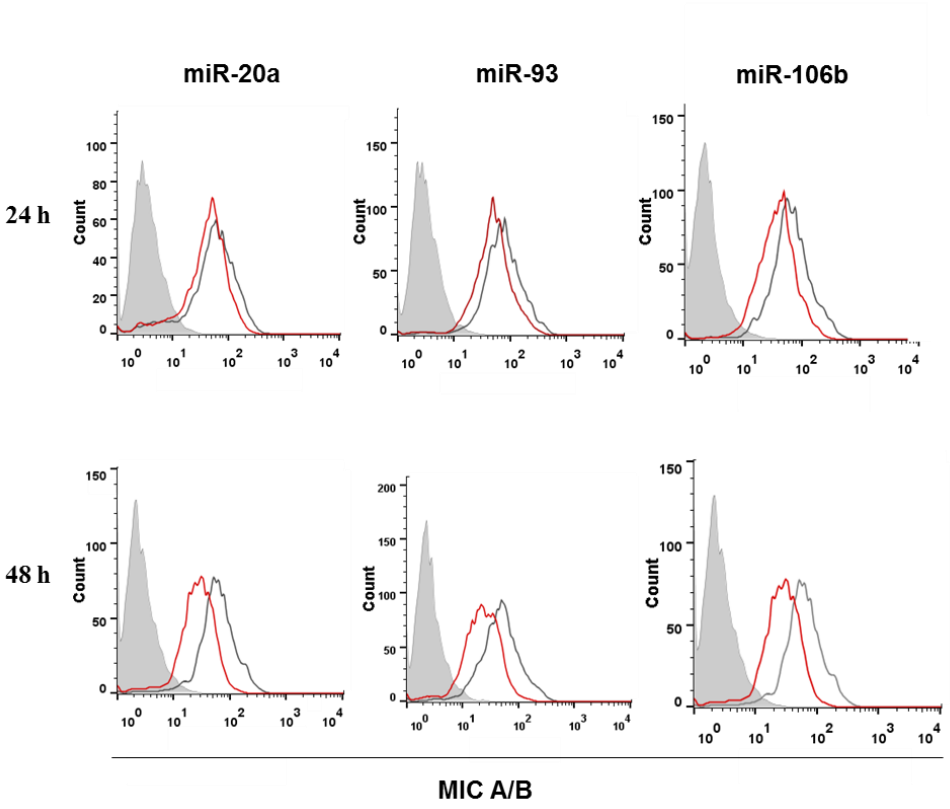
### MiR-20a, miR-93, and miR-106b regulate MICA and MICB expression in OS cells.

The role of miR-20a, miR-93, and miR-106b in regulating MICA and MICB expression was examined in OS cell lines. LM7 and KRIB cells were transiently transfected with the miR-20a, miR-93, and miR-106b mimics or control miRNA. These cell lines were chosen for the study because of high levels of MICA and MICB expression on the surface. Overexpression of miRNA in transfected cell lines was confirmed by quantitative RT-PCR (figure10A and 10B).

Cells were harvested 24 and 48 hours after transfection and MICA and MICB expression levels were analyzed by flow cytometry. Consistent with the studies done by Stern-Ginossar et al., our data showed that these miRNAs down-regulated MICA and MICB expression in LM7 and KRIB cells 24 and 48 hours post transfection (figure 10C and 10D). These results supported the role of miRNAs i.e miR-20a, miR-93, and miR-106b in regulating MICA and MICB expression in OS cells.


A
LM7 cells
miR-20a
miR-93
miR-106b
24 h
48 h
Relative fold increase in miR-20a
Relative fold increase in miR-93
Relative fold increase miR-106b
Relative fold of miR-93 mRNA
Relative fold increase in miR-106b
Ctrl
miR-20a
miR-93
miR-106b
B
KRIB cells
miR-20a
miR-93
miR-106b
6 h
12 h
Relative fold increase in miR-20a
Relative fold increase in miR-93
Relative fold increase in miR-106b
Ctrl
miR-20a
miR-93
miR-106b
mir-106b

C


miR-20a
miR-93
miR-106b
24 h
48 h
Count
MIC A/B

D

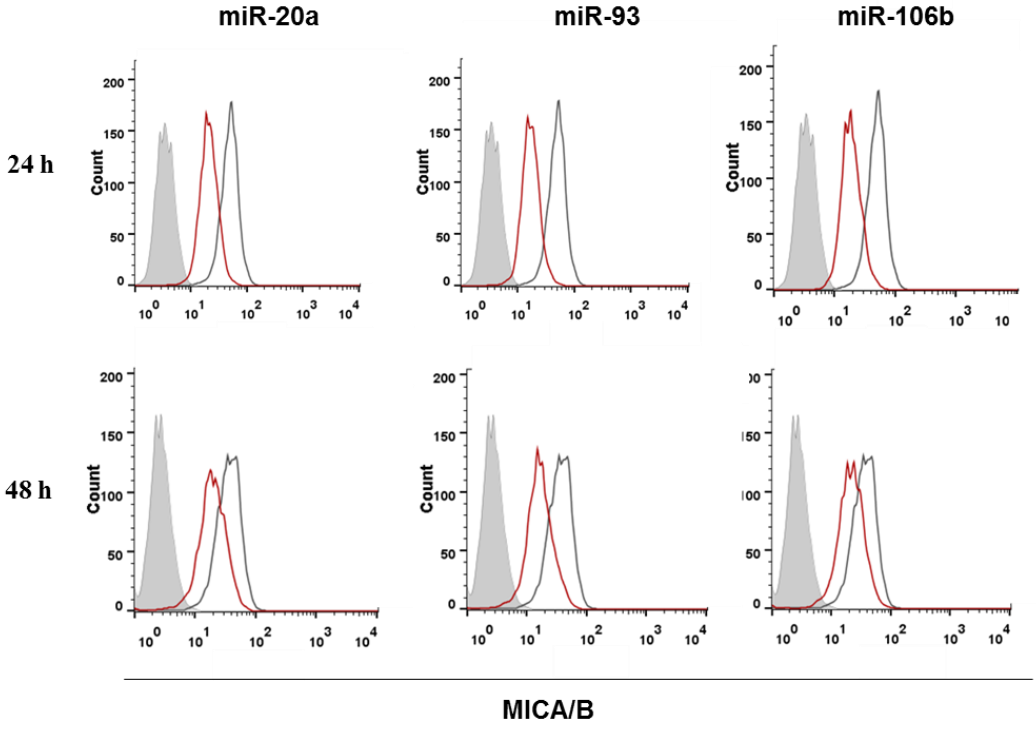
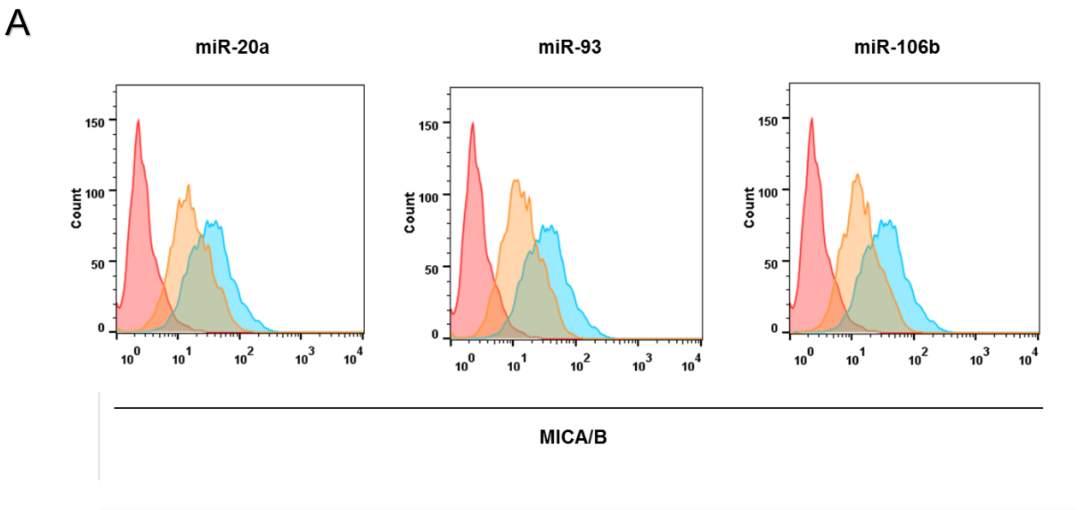
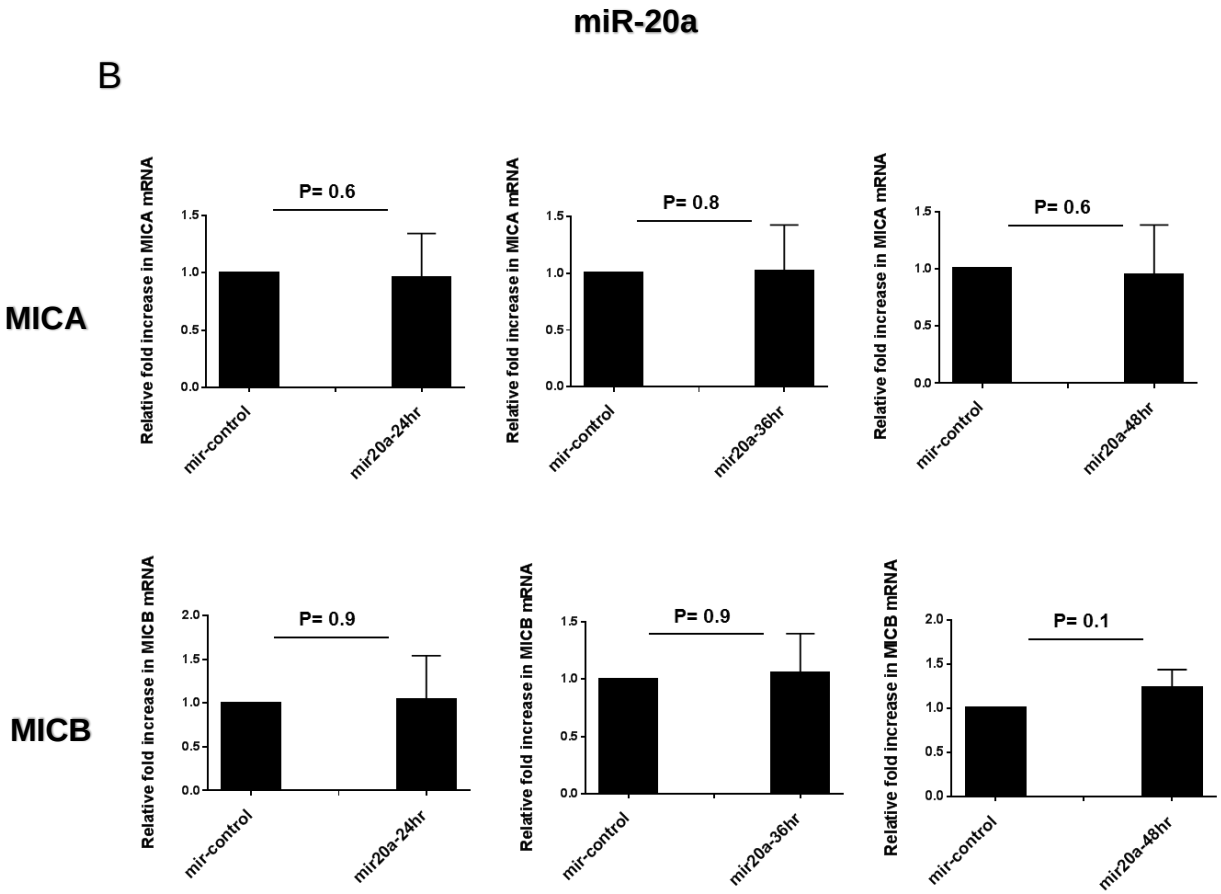

miR-20a
miR-93
miR-106b
24 h
48 h
Count
MICA/B

**Figure 10. miR-20a, miR-93, and miR-106b down-regulate MICA/B expression in OS cells.** miR-20a, miR-93, or miR-106b mimics or control miRNA were transiently transfected into LM7 and KRIB cell lines that express high levels of MICA/B. (A) Overexpression of miRNA in LM7 cells was confirmed by quantitative RT-PCR after 24 and 48 hours (B) Overexpression of miRNA in KRIB cell line was confirmed by quantitative RT-PCR 6 and 12 hours post-transfection. (C) LM7 and (D) KRIB cell lines were analyzed for MICA and MICB expression by flow cytometry 24 and 48 hours after miRNA transfection. Red lines: cell transfected with miRNA mimics; gray lines: cells transfected with control miRNA. Bars show mean +/- S.E.M, n=3.

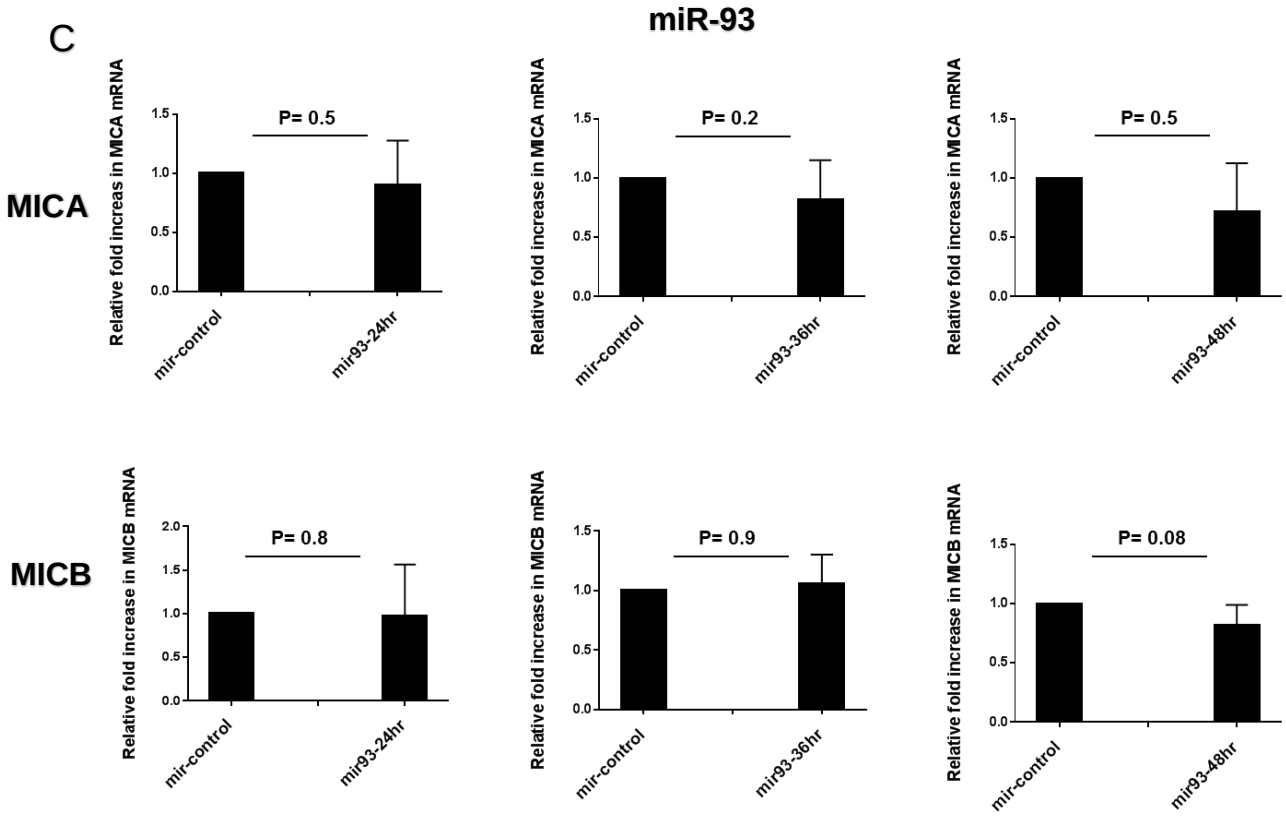
**Down-regulation of MICA and MICB expression by miR-20a, miR-93, and miR-106b is not controlled by MICA and MICB mRNA degradation.**

mirRNAs can induce gene silencing by either inhibition of mRNA translation or by promoting mRNA degradation and both mechanisms result in reduced protein expression level. We over-expressed miR-20a, miR-93, or miR-106b in LM7 cells and measured MICA and MICB mRNA levels. Our results showed no significant changes in MICA and MICB mRNA levels in LM7 transfected with miR-20a, miR-93, or miR-106b (figure 11B-11D). These findings suggest that MICA and MICB down-regulation in LM7 by miRNAs may be secondary to the inhibition of mRNA translation rather than the degradation of the mRNA.


A
miR-20a
miR-93
miR-106b
Count
MICA/B


miR-20a
B
MICA
MICB
Relative fold increase in MICA mRNA
Relative fold increase in MICB mRNA
P= 0.6
P= 0.8
P= 0.6
P= 0.9
P= 0.9
P= 0.1
mir-control
mir20a-24hr
mir20a-36hr
mir20a-48hr

C


miR-93
MICA
MICB
Relative fold increas in MCA mRNA
Relative fold increase in MCA mRNA
Relative fold increase in MICB mRNA
P= 0.5
P= 0.2
P= 0.5
P= 0.8
P= 0.9
P= 0.08
mir-control
mir93-24hr
mir93-36hr
mir93-48hr

D

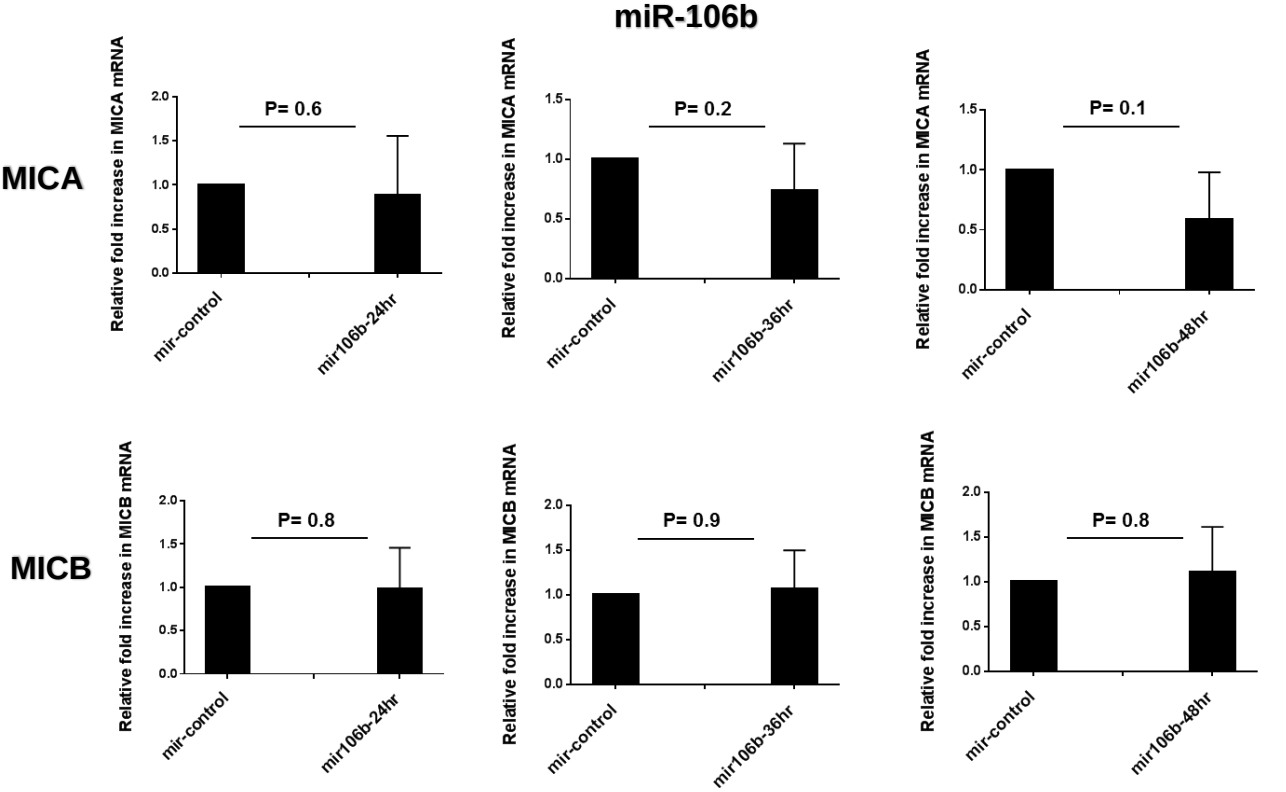

miR-106b
MICA
MICB
Relative fold increase in MICA mRNA
Relative fold increase in MICB mRNA
P= 0.6
P= 0.2
P= 0.1
P= 0.8
P= 0.9
P= 0.8
mir-control
mir106b-24hr
mir106b-36hr
mir106b-48hr

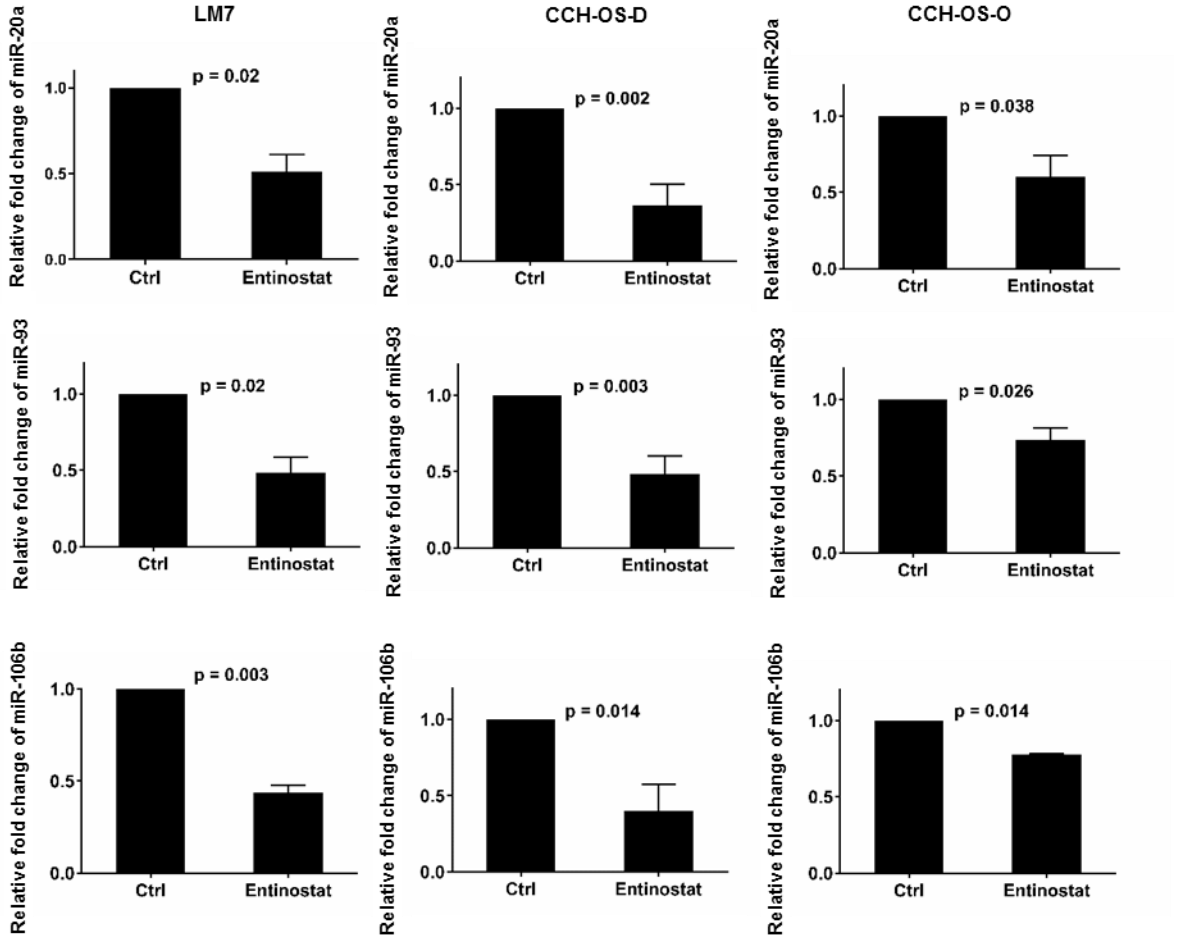
**Figure 11. miR-20a, miR-93, and miR-106b decreases MICA and MICB expression in OS cell lines but has no effect on total mRNA levels.** (A) miR-20a, miR-93, or miR-106b mimics (orange lines) or control miRNA (blue lines) were transiently transfected into LM7.Cells were collected 48 h following transfection and were analyzed by flow cytometry for MICA/B expression. (B-D) Total RNA was extracted 24, 36, and 48 h following miRNA transfection and MICA and MICB mRNA levels were measured by using quantitative RT-PCR. Bars show mean +/- S.E.M, n=3.

**Entinostat up-regulates MICA and MICB expression by down-regulating miR-20a, miR-93, and miR-106b in OS cells *in vitro* and *in vivo.***

Having shown previously that entinostat significantly increased the expression of MICA/B (chapter 2, figure 1) and that miR-20a, miR-93, and miR-106b down-regulated MICA/B expression in OS cell lines (chapter 5, figure 10C and 10D), next, we hypothesized that entinostat indirectly controls MICA/B expression by down-regulating miR-20a, miR-93, and miR-106b in OS cells. To test this hypothesis, three OS cell lines LM7, CCH-OS-D, and CCH-OS-O were treated with 2 μM entinostat for 48 hours. Quantitative RT-PCR analysis revealed that all three miRNAs were significantly decreased in the entinostat treated OS cell lines compared to the control samples (figure 12A). We also sought to investigate whether entinostat regulates MICA and MICB expression in OS lung metastasis by reducing miR-20a, miR-93, and miR-106b using *in vivo* mouse model. Approximately $2 \times 10^6$ LM7 cells, a human OS cell line that forms direct metastasis in the lung, were injected intravenously into nude mice. After visible nodule formation in the lung, the mice were treated with 5 mg/kg entinostat by oral/gavage three times a week for 2 weeks. Control mice were treated with DMSO. The mice were the euthanized, total RNAs were extracted from lung nodules, and MICA and

MICB mRNA levels as well as miRNA expression were evaluated by quantitative RT-PCR. Results indicated that 5 mg/kg entinostat significantly increased MICA and MICB mRNA levels (figure 12B) and decreased miR-20a, miR-93, and miR-106b in OS lung metastasis (figure 12C). These *in vitro* and *in vivo* results indicate that entinostat treatment regulates MICA and MICB expression by down-regulating miR-20a, miR-93, and miR-106b expression in OS cells and also in lung metastasis.

A



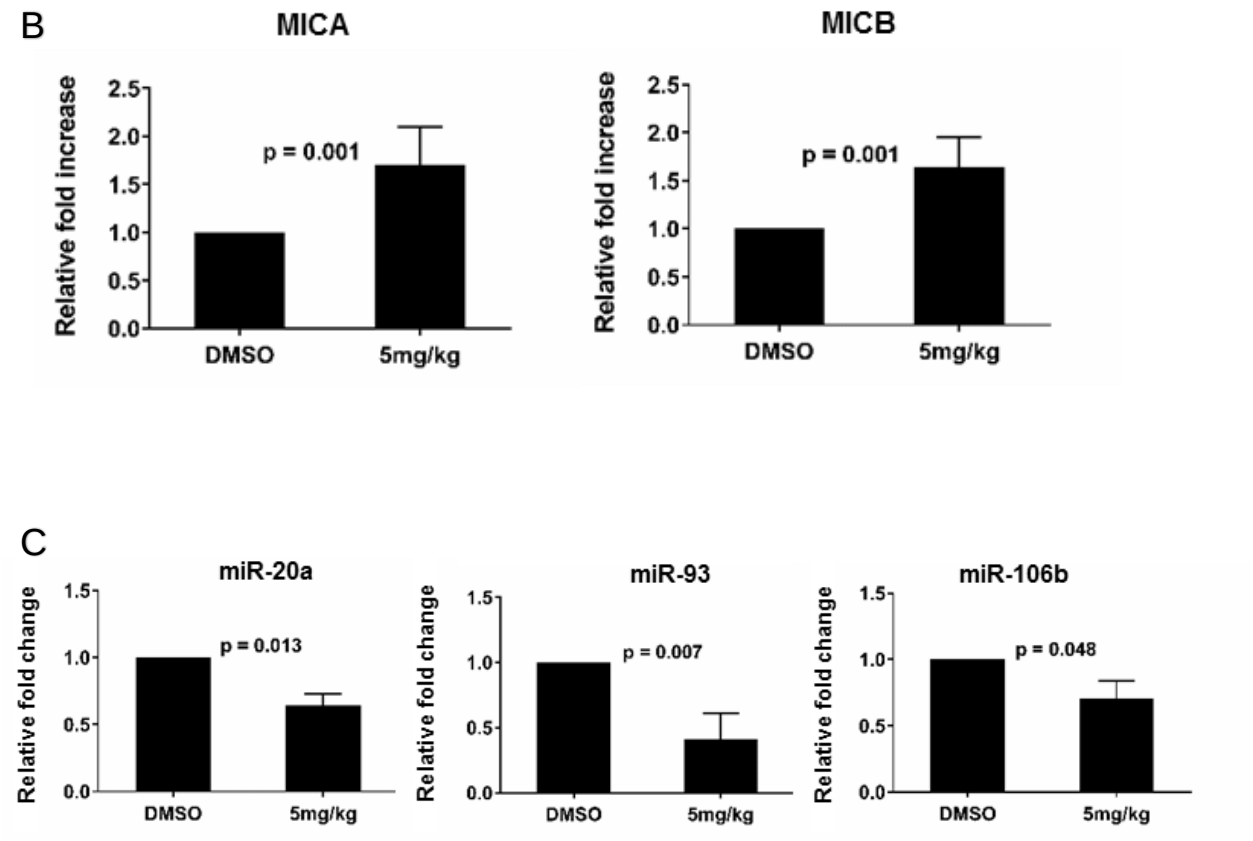



**Figure 12. Entinostat regulates MICA and MICB expression by down-regulating miR-20a, miR-93, and miR-106b expression.** (A) LM7, CCH-OS-D, and CCH-OS-O cell lines were treated with 2 μM entinostat for 48 h. Total RNAs were isolated with the use of Trizol reagent. Reverse Transcription was performed using TagMan miRNA reverse transcription kit. The resulting cDNA was subjected to PCR amplification using primers specific for mir-20a, mir-93, or mir106b. (B, C) Mice were injected with $2\times 10^6$ LM7 cells intravenously. Following visible nodule formation in the lung, mice were treated with 5mg/kg entinostat three times a week for two weeks. Control mice received DMSO instead. Thereafter, mRNA was extracted from lung nodules and (B) MICA and MICB mRNA levels and (C) miRNA expression were evaluated by quantitative RT-PCR. Data shown mean $\pm$ S.E.M.

## SUMMARY

In this chapter we demonstrated that expression of MICA and MICB is regulated by miR-20a, miR-93, and miR-106b in OS cells lines. We also showed that miR-20a, miR-93, and miR-106b down-regulate MICA and MICB expression without altering the mRNA levels, suggesting that the effect is through the inhibition of MICA and MICB mRNA translation. Our data support the conclusion that entinostat increases MICA and MICB expression by down-regulating miR-20a, miR-93, and miR-106b.

# Chapter 6

## NK cell therapy in combination with oral administration of entinostat in mice with OS pulmonary metastasis

## RATIONALE

In the previous chapters we showed that entinostat up-regulates MICA/B expression in OS cells *in vitro* (figure 1) and in OS lung metastasis *in vivo* (figure 12B), and also enhances ULBP2/5/6, and CD155 in OS cell lines *in vitro* (figure 1). Increased expression of NK cell ligands on OS cells should enhance NK cell-mediated cytotoxicity as we showed in our *in vitro* experiments. Accordingly, we hypothesized that combination therapy using entinostat with NK cells would enhance NK cell therapeutic effect in our mouse model with established OS lung metastasis. It has been reported previously in a mouse model (NOD.CgRag1$^{tm1Mom}$Prf1$^{tm1Sdz}$/SzJ) with fibrosarcoma lung metastasis, that treatment with NK cells and entinostat significantly lowered tumor burden. (58). Further, in a xenograft orthotopic OS model (NSG mouse), mice treated with NK cells and IL-2 (10,000 IU IP injection/mouse) had reduced bone damage, lower tumor burden in the tibia, and longer survival rates with no lung metastasis when compared to control group (45).

## RSULTS

### Five mg/kg is the lowest dose of entinostat that significantly increases MICA and MICB mRNAs in OS lung metastasis.

Before initiating our *in vivo* study with combination NK cells and entinostat therapy, we first wished to determine a sub-therapeutic dose of entinostat that can significantly increase the expression of NK cell ligands on the OS lung metastasis.

Approximately, 2 × $10^6$ LM7 cells were injected via tail vein into nude mice. After visible nodule formation in the lungs, the mice were treated with entinostat of 2.5, 5, or 10 mg/kg by oral/gavage three times a week for 2 weeks (figure 13). Our group had previously demonstrated that 20 mg/kg of entinostat has a therapeutic effect against OS lung metastasis and inhibits tumor growth (79). Therefore, the highest dose of the drug we examined was 10 mg/kg. At the end of the treatment, total RNA was extracted from the lung tumors and MICA and MICB mRNA levels were evaluated by using quantitative RT-PCR. The results demonstrated that both 5 and 10 mg/kg entinostat significantly enhanced the MICA and MICB mRNA levels; however, 2.5 mg/kg of entinostat did not significantly increase the MICA and MICB mRNAs (figure 14). Thus, we chose to use 5 mg/kg entinostat in our animal study as the lowest dose that significantly increased MICA and MICB ligands in OS lung metastasis.

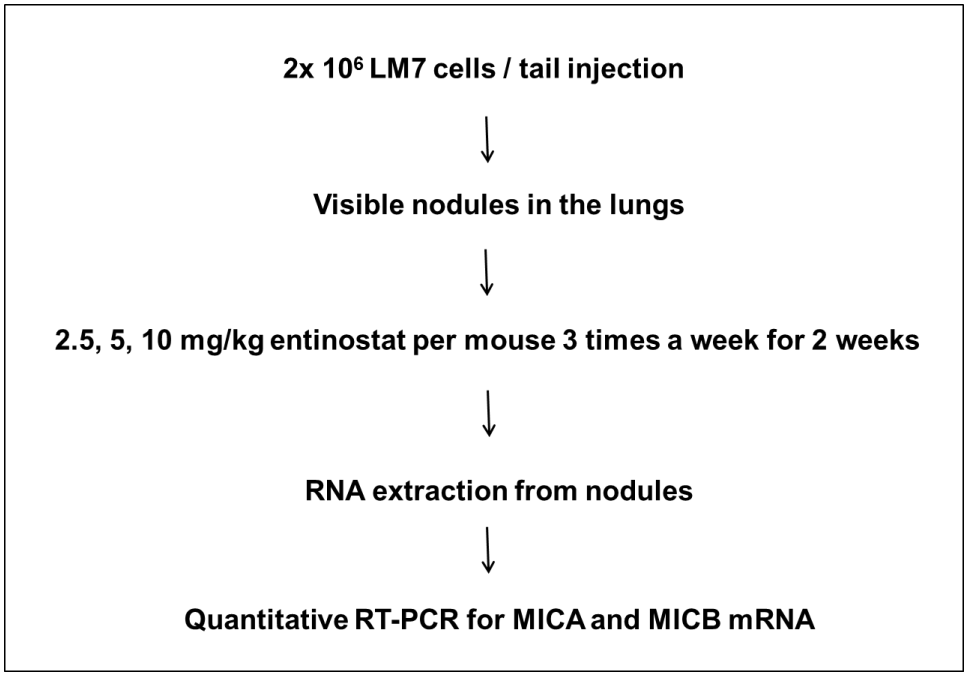


**Figure 13. Schematic of mice experimental design to determine sub-therapeutic dose of entinostat for increasing MICA and MICB mRNA levels.** Approximately 2× $10^6$ LM7 cells in 0.2 mL of PBS were injected into nude mice through the tail vein. Following visible nodule formation in the lung, mice were treated with 2.5, 5, 10 mg/kg entinostat by oral/gavage 3 times a week for 2 weeks. Mice were sacrificed and total RNA was extracted from lung nodules. MICA and MICB mRNA levels were determined by quantitative RT-PCR.

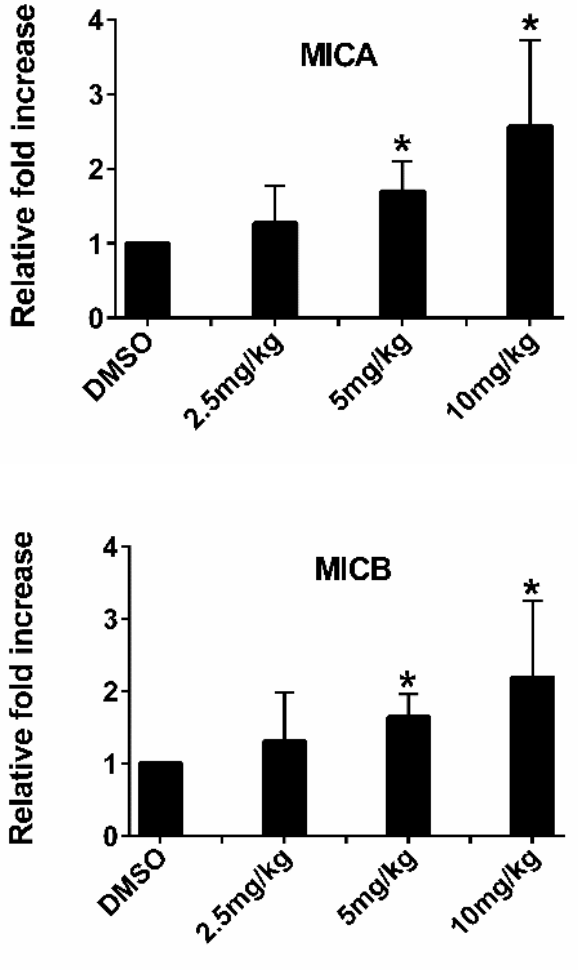



**Figure 14. Determining the lowest dose of entinostat that significantly increases MICA and MICB mRNAs in OS lung metastasis.** 2× $10^6$ LM7 cells suspended in 0.2 mL of PBS was injected into nude mice. Following visible nodule formation in the lungs, mice were treated with entinostat concentrations of 2.5, 5,

or 10 mg/kg by oral/gavage 3 times a week for 2 weeks. At the end of the treatment nodules were removed from the lungs and total RNA was extracted and MICA and MICB mRNA levels were determined by quantitative RT-PCR. Data was shown as mean ± S.E.M, n=5.

**NK cells used for immunotherapy were highly activated and functional.**

Human NK cells were isolated from normal donors' buffy coats and were expanded *in vitro* for 4 weeks using genetically engineered K562 supplemented with 50 IU/ml recombinant human IL-2. K562 cells were used as an artificial antigen-presenting cell (aAPC) to propagate NK cells *in vitro.* The genetically engineered K562 expressed IL-21, CD86 (B7-2), CD64 (FccRI), and CD137L (4-1BBL).

Expanded NK cells were depleted for $CD3^+$ T cells and had basic phenotype $CD3^-$ $CD56^+$ (Fig 15A). Their receptor expression i.e. NKG2D, CD16 and functional activity were also evaluated before being used *in vivo*. All expanded NK cells were ≥ 95% and ≥ 80% positive for NKG2D and CD16, respectively. The expanded NK cells were also tested for their cytotoxicity against LM7 cells before Infusing into mice. At a ratio of 0.3:1 and 10: 1 (effector: target cell ratio), NK cell-mediated cytotoxicity against LM7 cell were 22± 5 and 93± 3 respectively (figure 15B). Our data demonstrated that NK cells from all donors were healthy and functional after four weeks of being expanded *in vitro*.

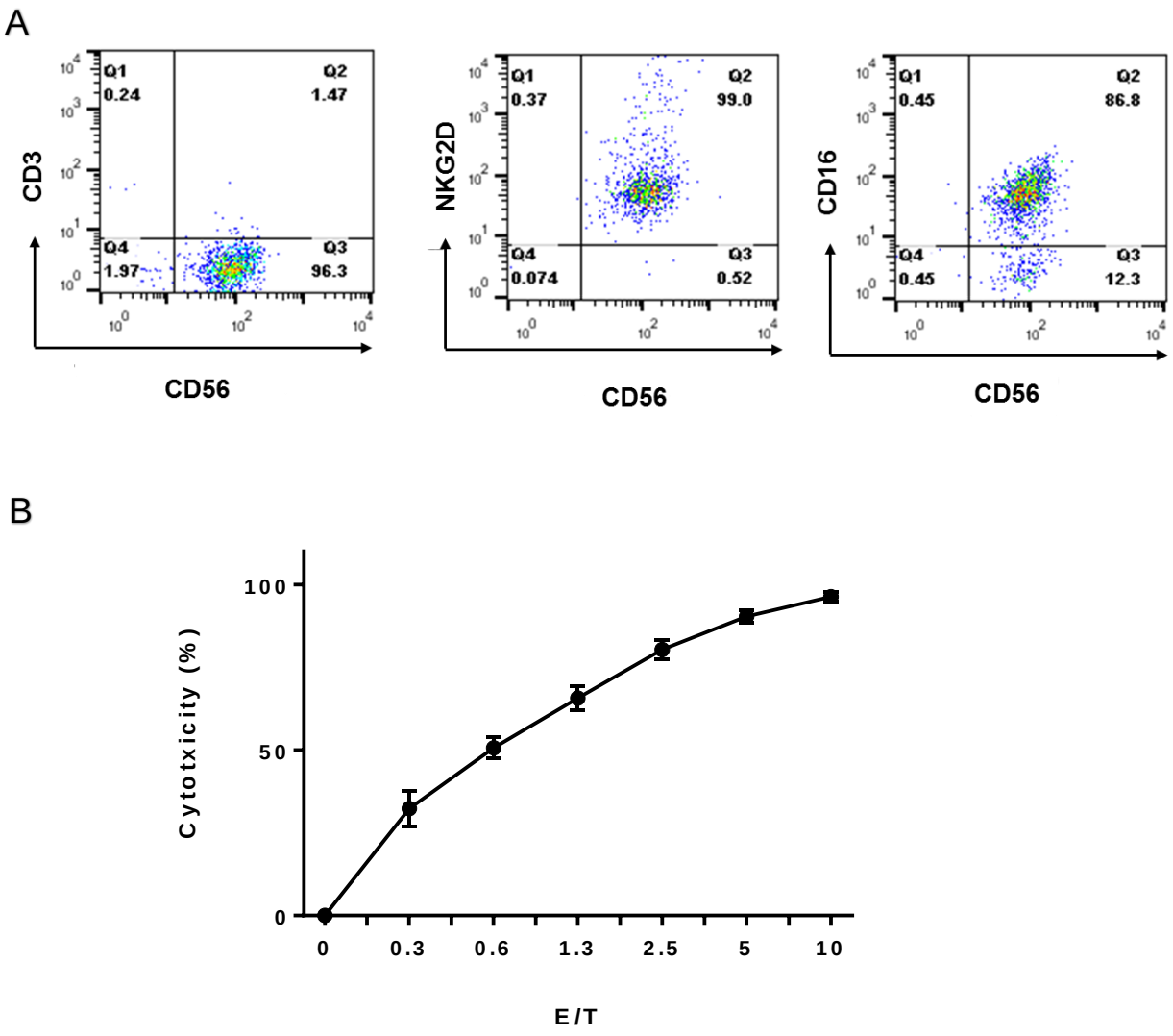



**Figure 15. *Ex vivo* expanded NK cells were fully activated and highly functional.** Human NK cells were purified from blood buffy coats and were expanded *in vitro* for 4 weeks using genetically engineered K562 and recombinant human IL-2. (A) NKG2D and CD16 receptor expression on NK cells after 4 weeks of expansion assessed by flow cytometry. (B) Representative *in vitro* cytotoxicity of expanded NK cells for OS cell LM7 by calcein release assay. Target cells labeled with 1 µM calcein-AM and incubated at 37ºC for 1 h. Cells were washed with RPMI media and co-cultured with *ex vivo* expanded NK cells in 96-well U-bottom plates at a ratio of 0.3, 0.6, 1.3, 2.5, 5, and 10 (effector: target cell ratio). Plates were incubated at 37ºC for 4 h. 100 µL of supernatant was transferred to a 96-well flat bottom plate. The plate was read at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

**NK Cell therapy in combination with entinostat treatment did not inhibit tumor growth in mice with osteosarcoma lung metastases.**

We demonstrated that entinostat sensitized OS cells to NK cell-mediated cytotoxicity by up-regulating the ligands for NK cell activating receptors, *in vitro*; therefore, we anticipated that adding entinostat to NK cell therapy would increase the therapeutic effect of NK cells for OS lung metastasis. To test this, we used our established OS pulmonary metastasis mouse model in which nude mice were injected with $2 \times 10^6$ LM7 cells via the tail vein. Pulmonary micrometastasis formation in 3 mice euthanized at week 5 was confirmed by H&E staining of paraffin-embedded lung section. After confirmation of micrometastsis formation, treatment was initiated in four groups of mice with DMSO, entotstat, NK cells+ DMSO, or entinostat+ NK cells for five weeks. A total of 12-16 mice were included in each group (figure 16).

We previously demostrated that up-regulated ligands on OS cells (MIC A/B, ULBP1, and ULBP2/5/6) by entinostat are stable for more than 24 h (Table 1), suggesting that there could be a window time between entinostat administration and NK cell infusion. Furthermore, we showed that 48 h but not 24 h of treatment with entinostat down-regulated NK cell receptor expression including: NKG2D, NKp30, NKp44, and NKp46 (figure 6). Therefore, we elected to administer entinostat and NK cells 24 h apart to minimize the effect of entinostat on NK cell receptor expression (Table 2).

At the end of treatment, mice were sacrificed and lungs were preserved for analysis. Contrary to our initial hypothesis, our findings showed that combining oral

administration of entinostat with NK cell therapy showed no therapeutic effect for OS lung metastasis. The combination therapy did not reduce either the number or the size of nodules in the lung (Figure 17,18).

| Groups | | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|---|---|
| Control | | DMSO | | | DMSO | | DMSO | |
| Entinostat | | Entinostat | | | Entinostat | | Entinostat | |
| NK cells | | DMSO | NK cells | | DMSO | NK cells | DMSO | |
| NK cells+ Entinostat | | Entinostat | NK cells | | Entinostat | NK cells | Entinostat | |

**Table 2. Experimental plan for combination therapy with entnostat and NK cells in mice with OS lung metastasis.** Mice were treated with entinostat at a concentration of 5 mg/kg of weight by oral/gavage three times a week (Monday, Thursday, and Saturday) for five weeks. 50 x $10^6$ NK cells were injected via tail vein two times in a week (Tuesday, and Friday) for 5 weeks.

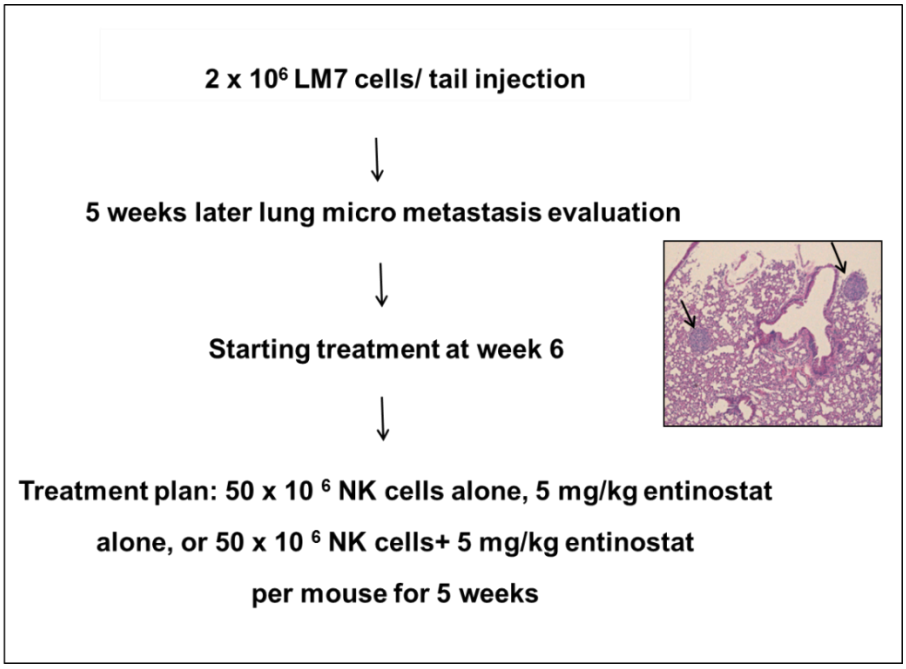


**Figure 16. Schematic of experimental plan for *in vivo* study.** Nude mice were injected with 2 × $10^6$ LM7 cells through tail vein. Presence of micrometastasis was confirmed by H&E staining five weeks after tumor cell infusion. Treatment was initiated on week six with DMSO, NK cells+ DMSO, entotstat, or entinostat+ NK cells for five weeks.

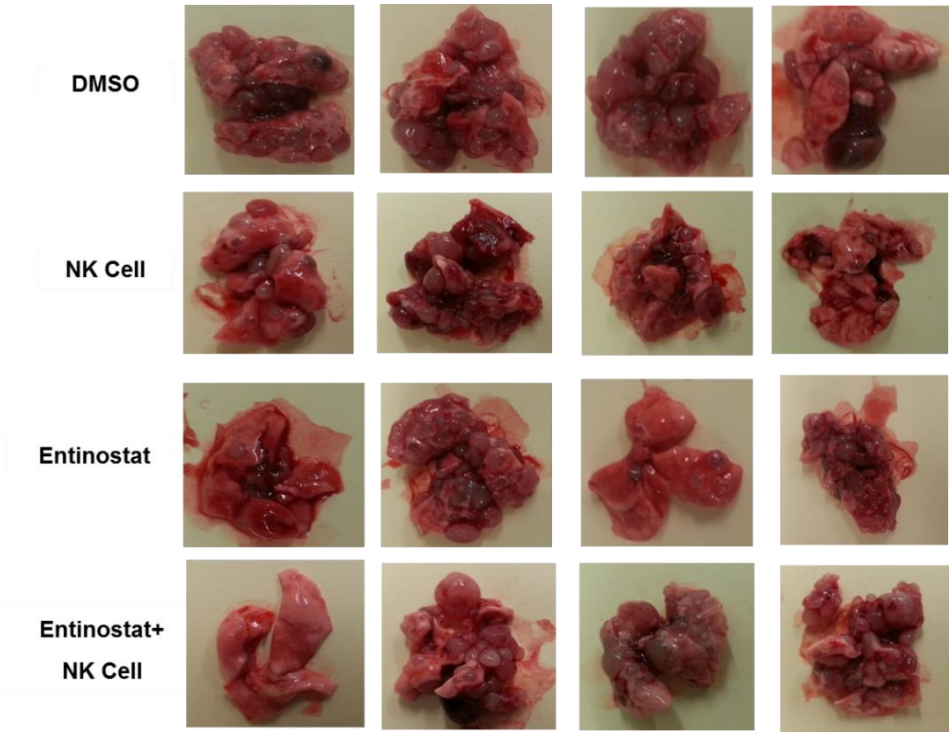



**Figure 17. Representative pictures of lungs from each mouse group after 5 weeks of treatment.** Nude mice were injected via tail vein with 2 × $10^6$ LM7 cells. Following micro metastasis formation mice were treated with DMSO, NK cells+ DMSO, entinostat, or entinostat+ NK cells for five weeks. Mice were sacrificed, their lungs were resected and analyzed for the presence of metastasis.

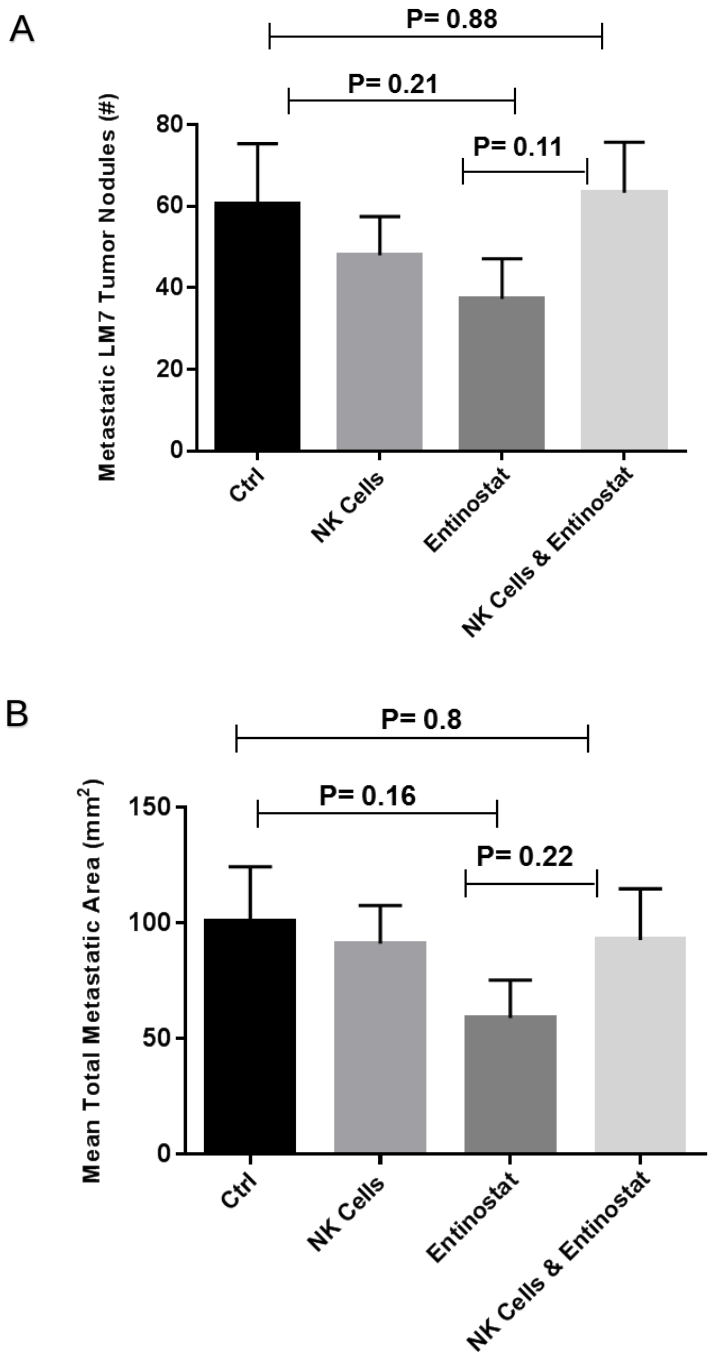


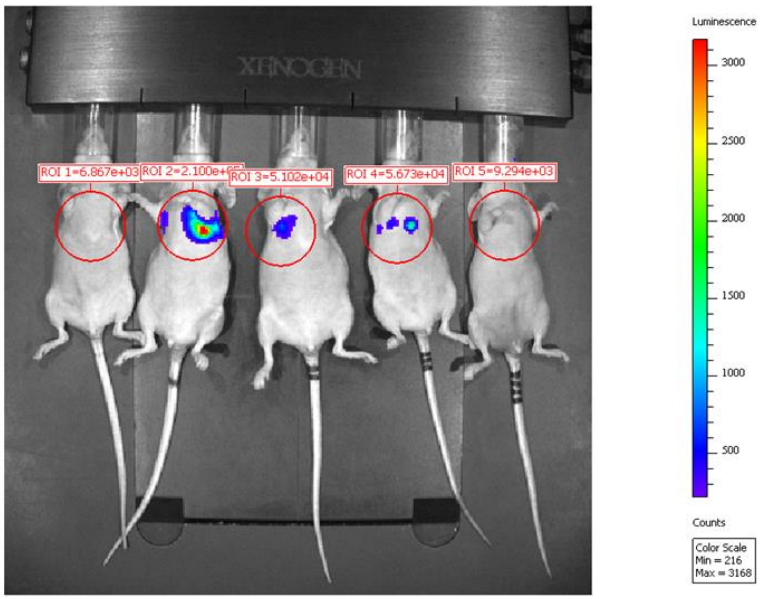

**Figure 18. Combination therapy with NK cells and entinostat did not have therapeutic effect in mice with stablished OS lung metastasis.** Nude mice were injected i.v. with $2 \times 10^6$ LM7 cells. Following micrometastasis formation mice were treated with DMSO, NK cells+ DMSO, entinostat, or entinostat+ NK cells for five weeks. Mice were sacrificed and lungs were resected. (A) Number of visible nodules in each mouse was quantified and the mean number of metastatic nodules was calculated for each group. (B) Diameter of each individual visible nodule was recorded and the area of all nodules in each lung was calculated. The results are

indicated as mean metastatic area of the nodules in each treatment group. Number of mice per groups= 12-16. Data= mean +/- S.E.M.

**The second *in vivo* study showed that NK cell therapy combined with the oral administration of entinostat had no therapeutic effect on OS lung metastasis.**

It is known that efficacy of immunotherapy for cancer is highly dependent on the effector to target cell ratio and that immunotherapy is mostly effective on minimal residual disease. The sooner we apply the immunotherapy the more chance of response. In order to monitor onset of micrometastasis formation in the lung more precisely, we used a genetically modified LM7 OS cell line that expresses firefly luciferase. We monitored tumor burden in the lungs by bioluminescent imaging (BLI). Mice were examined on a weekly basis and when ≥ 75% of the mice were positive for micrometastasis, they were evenly distributed into four groups and were treated with DMSO, entinostat, NK cells+ DMSO, or entinostat+ NK cells for five weeks (n=12/ group). Tumor burden was assessed by BLI the day before treatment and 5 weeks after treatment (figure 19). Relative fold increase in the flux was compared between the groups. Similar to our first study, our results indicated that combination therapy with NK cells and entinostat had no therapeutic effect on OS lung metastasis and did not reduce the tumor burden in the lung (figure 20). Furthermore, overall survival of mice treated with entinostat and NK cells was not improved (figure 21). No significant differences were observed in overall survival between mouse groups.

**Before treatment**




**After 5 weeks of treatment**

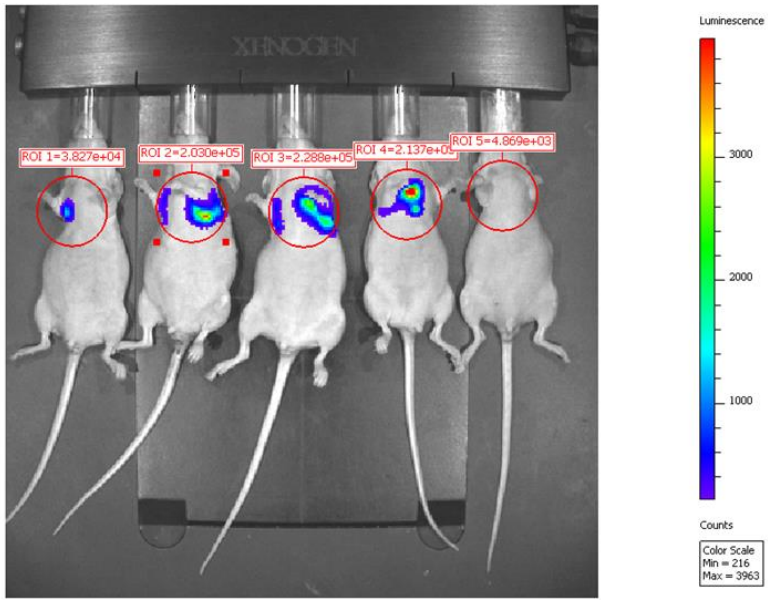



**Figure 19. Tumor burden assessed by BLI before (A) and after five weeks of treatment (B).** Nude mice were injected via tail vein with 2 × 106 LM7-Luc cells. Mice were monitored for micro metastasis formation in the lung every week. When micrometastasis were observed in 75% of mice, they were evenly distributed into four groups. Treatment was initiated with DMSO, NK cells+ DMSO, entinostat, or entinostat+ NK cells for five weeks. Before treatment and at the end of treatment tumor Burden was assessed by BLI using the IVIS spectrum system.

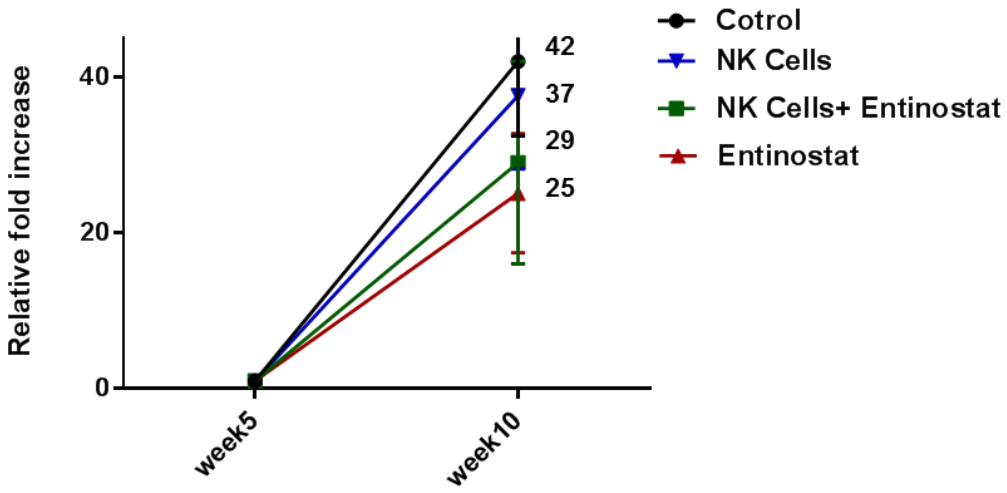



**Figure 20. Combination therapy with NK cells and entinostat did not have a therapeutic effect on mice with established OS lung metastasis.** Nude mice were injected via tail vein with 2 × $10^6$ LM7-Luc cells. Following micrometastasis formation treatment was initiated with DMSO, NK cells+ DMSO, entinostat, or entinostat+ NK cells for five weeks. Tumor burden was evaluated by BLI before treatment and after 5 weeks of treatment and then mean relative fold increase in the flux was calculated for each group.

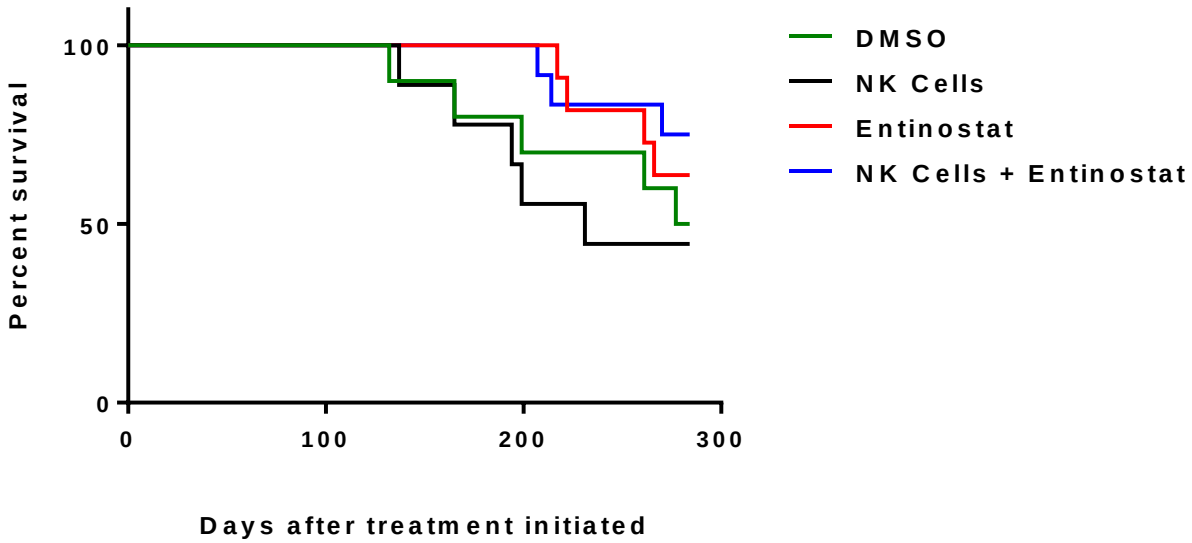
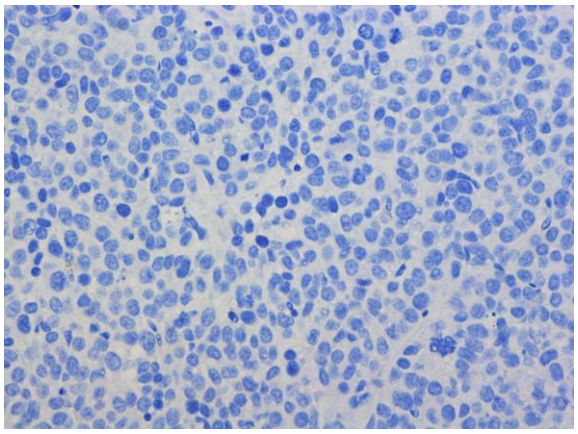



**Figure 21. Overall survival of mice treated with entinostat and NK cells were not improved.** Nude mice were injected i.v. with 2× $10^6$ LM7-Luc cells. Following micrometastasis formation mice were treated with DMSO, NK cells+ DMSO, entinostat, or entinostat+ NK cells for five weeks. Long term survival was assessed from the day that treatment was initiated until the mice died.

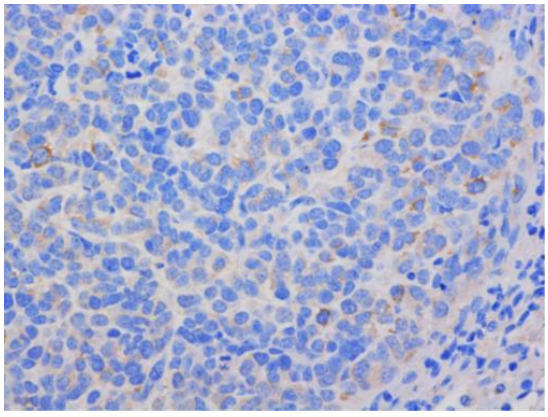
**Entinostat increased MICA/B expression on OS lung metastasis.**

To determine whether entinostat up-regulated MICA/B expression on OS lung metastasis, tumor sections from our *in vivo* experiment were analyzed by immunohistochemistry staining for MICA/B. Our results showed that MICA/B expression was significantly up-regulated in mice treated with entinostat or entinostat+ NK cells (figure 22). Before, we showed that entinostat significantly increases MICA and MICB mRNAs in OS lung metastasis (figure 14). These results are consistent with our *in vitro* findings (figure 1, 2).

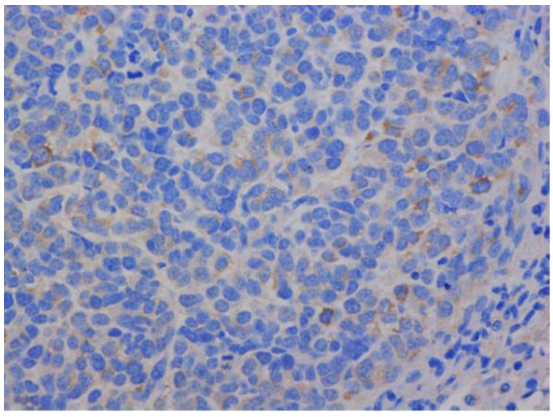
**Negative control**



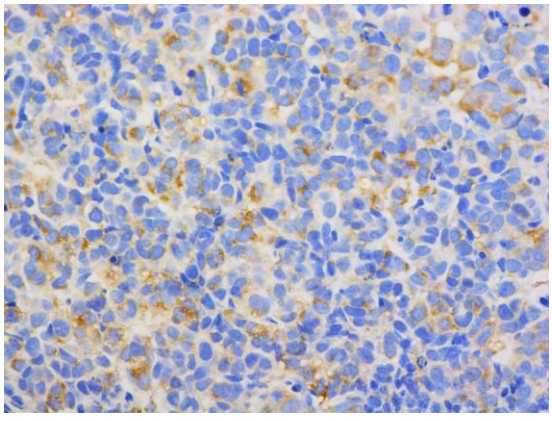
**DMSO**



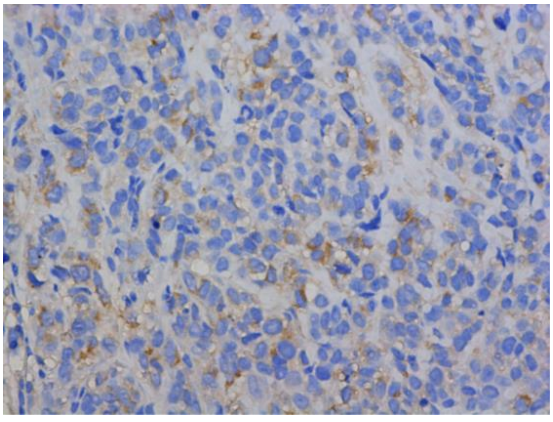
**NK cells**



**Entinostat**



**Entinostat+ NK cells**



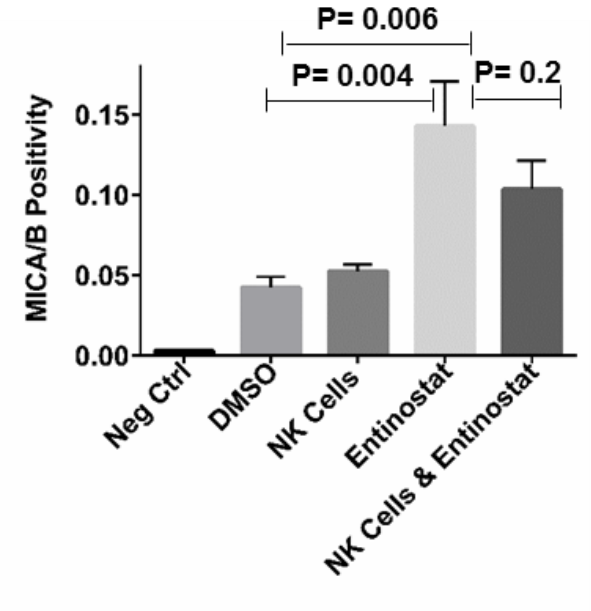



**Figure 22. Entinostat increased MICA/B expression on OS lung metastasis. (**A) Paraffin-embedded lung tissues were analyzed for MICA/B expression using immunohistochemistry staining. (B) Mean PD-L1 positivity was calculated in 5 random fields per section using the Simple PCI software.

**Infused NK cells can infiltrate into the mouse lung but do not penetrate into the lung tumor nodules.**

In order for NK cells to have a therapeutic effect, they must reach the target organ and penetrate into the tumor nodules. Having shown that combining NK cell therapy and oral administration of entinostat had no therapeutic effect in mice with OS lung metastasis, we first examined whether injected NK cells can traffic to the lungs. For addressing this question, nude mice were injected via the tail vein with 50 x $10^6$ CM-DiL-labeled NK cells. At 50 min and 24 h after NK cell injection, the

mice were euthanized and lungs were removed. Frozen sections of the lungs were examined under a fluorescent microscope. High numbers of NK cells were present in the lung 50 min after injection; however, by 24 h after injection there were very few NK cells left in the lung (figure 23). This data confirmed that NK cell can traffic into the mouse lungs but either exit or die within 24 h.

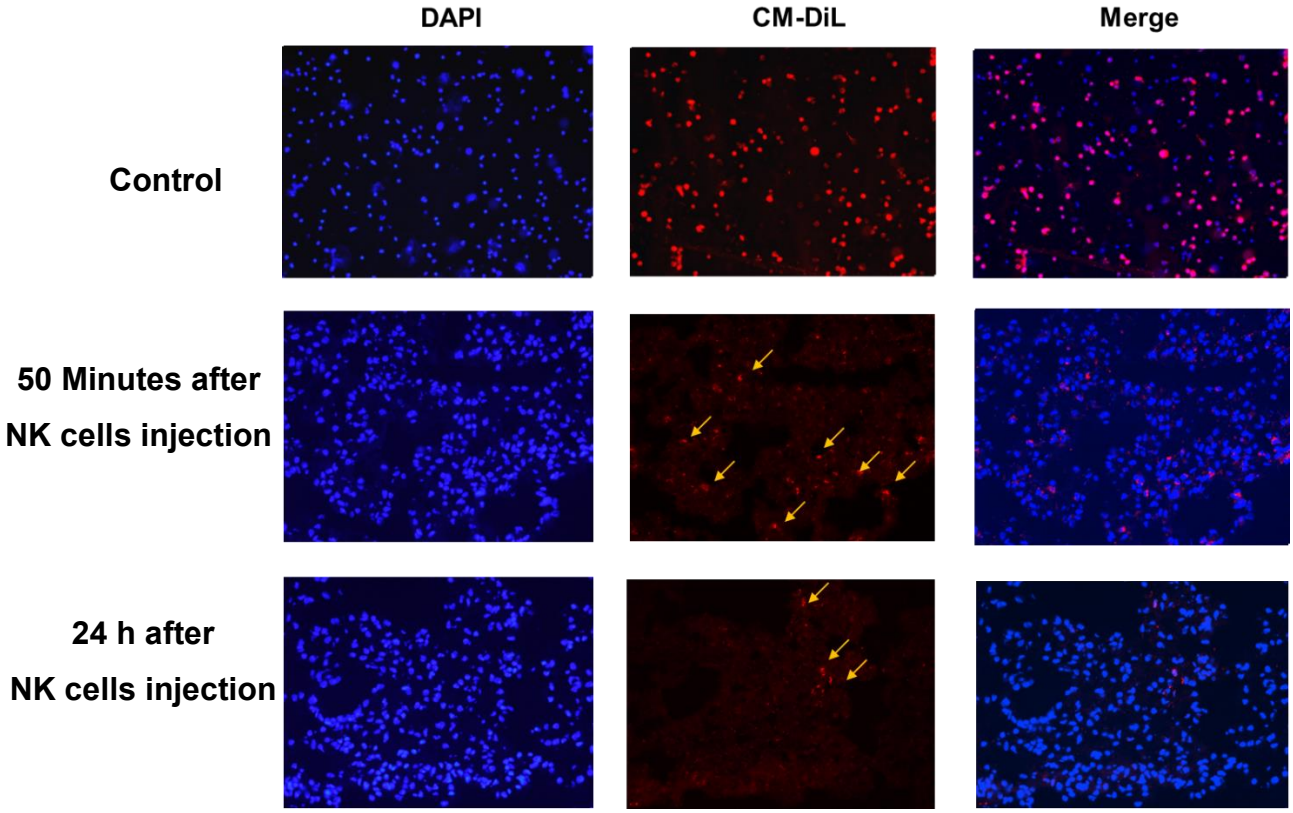



**Figure 23. Infused NK cells can infiltrate into the mouse lungs.** Nude mice were injected via tail vein with $50x10^6$ CM-DiL-labeled NK cells. Fifty minutes and 24 h post injection, mice were sacrificed and lungs were resected. The frozen sections of the lung tissues were stained for nucleus using Hoechst 33342 nucleic acid stain. *In vitro* NK cells labeled with CM-DiL were examined under the microscope to confirm that NK cell staining had been successful (top row).

We further investigated whether following migration to the lungs, NK cells also penetrated into the OS lung metastasis. Tumor sections from our *in vivo* experiment were analyzed by immunofluorescence staining for NK cells. As shown in figure 24, NK cells were observed at the border of the nodules, but not inside them. Therefore, the lack of therapeutic effectiveness of combination therapy may be due to the inability of the NK cells to infiltrate into OS lung metastasis.

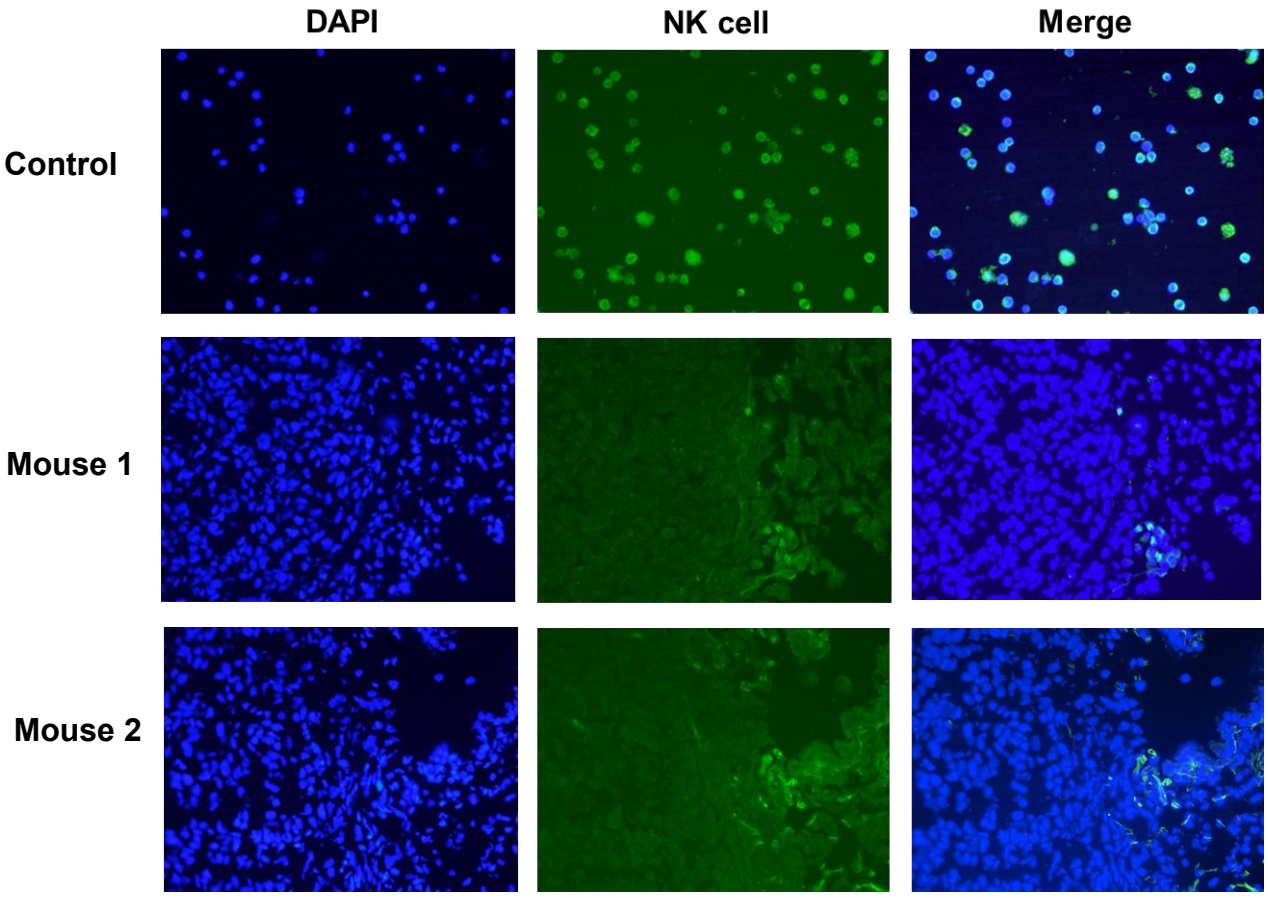



**Figure 24. Infused NK cells did not infiltrate into the OS lung metastasis.** Representative pictures of NK cells in OS lung metastasis from mice treated with NK cells in combination with entinostat. Frozen sections of the tumor nodules were stained for the NK cell marker NKp46 and was examined using a fluorescent microscopy. *Ex vivo* expanded NK cells were stained for the NKp46 marker to confirm that staining had been successful (top row).

**Entinostat increased PD-L1 expression on OS cells *in vitro*.**

To further investigate why our combination therapy was not effective against lung metastasis, we examined whether entinostat treatment increased expression of programmed death-ligand 1 (PDL-1) on OS cells. PDL-1 is a ligand for the programmed death 1 (PD-1) receptor which is an immune inhibitory receptor expressed on immune cells, including NK cells. Interaction between PD-1 and PDL-1 induces an immune suppressive effect on immune cell and allows tumor cells to escape from immune cell mediated killing (11).

LM7 and CCH-OS-D cells were treated with various doses of entinostat for 24 h and expression of PDL-1 was evaluated by flow cytometry. Results demonstrated that PDL-1 expression is upregulated on LM7 and CCH-OS-D cells in a dose dependent manner, suggesting a mechanism by which OS metastasis can escape from NK cell therapy (figure 25).

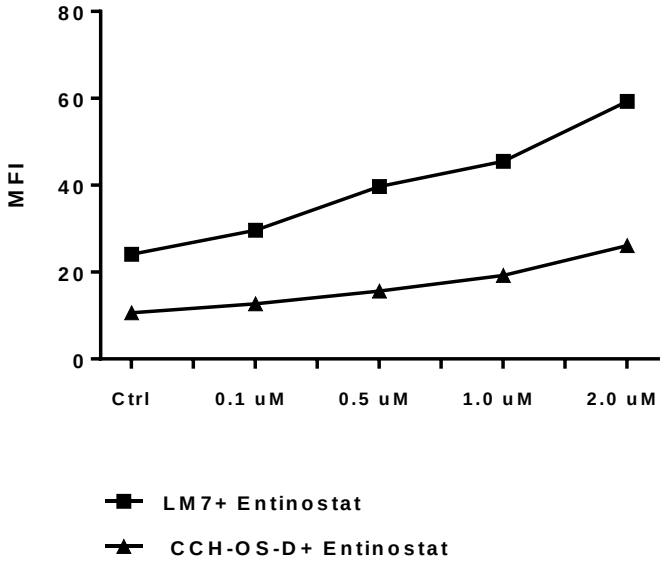


**Figure 25. Entinostat increases PD-L1 expression on OS cells in a dose dependent manner.** LM7 and CCH-OS-D cells were treated with 0.1, 0.5, 1.0, or 2.0 µM entinostat for 24 h. Cells were stained for PD-L1 and were analyzed by flow cytometry. Untreated cells were used as control.

### *Ex vivo* expanded human NK cells express PD-1.

To verify that the NK cells used for the animal studies expressed PD-1, we purified human NK cells from heathy donors' buffy coats and expanded them *in vitro* for three weeks, as described earlier. PD-1 expression was then analyzed by flow cytometry. As it is shown in figure 26, approximately 6-8% of *ex vivo* expanded NK cells express PD-1.

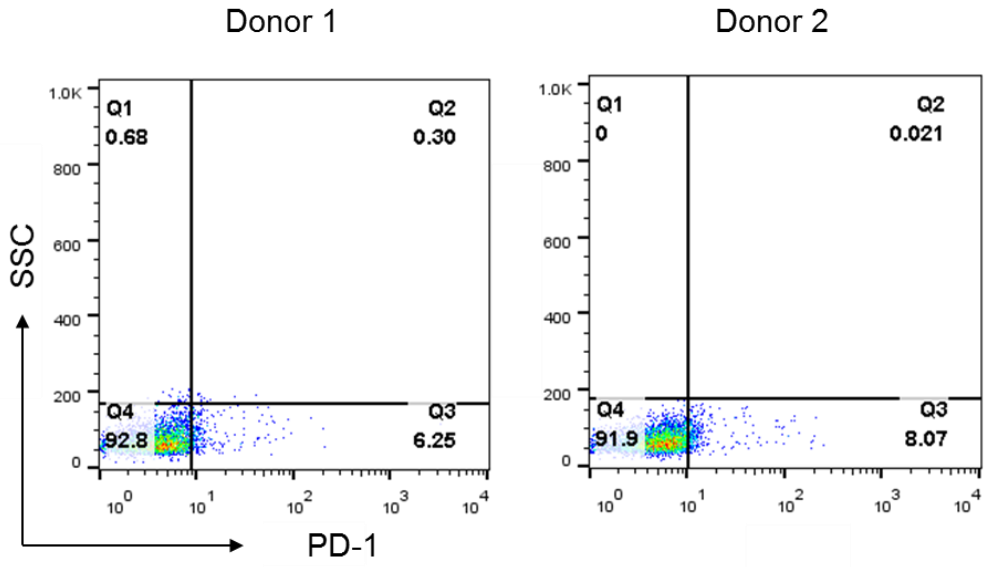



**Figure 26. *Ex vivo* expanded human NK cells express PD-1.** Flow cytometry analysis for PD-1 expression on expanded NK cells. Human NK cells were purified from buffy coats and were expanded *in vitro* for three weeks using genetically engineered K562 and recombinant human IL-2. Cells were stained for PD-1 and expression of receptor was analyzed by flow cytometry.

## SUMMERY

Our *in vivo* findings demonstrated that combination therapy with entinostat and NK cell had no therapeutic effect on OS lung metastasis in our mouse model. This result is in contrast with our *in vitro* data showing that OS cells are sensitive to NK cell killing and that entinostat enhances OS cell susceptibility to NK cell-mediated cytotoxicity. We also showed that although infused NK cells infiltrate into the lung, they are not able to penetrate into the tumors. Further, by showing that entinostat increased PD-L1 expression on OS cells *in vitro*, we proposed an additional mechanism of escape by tumor cell by interacting with the PD-1 receptor on NK cells and inducing inhibitory signals.

# Chapter 7

# Discussion: Implications of Results and Future Directions

There has been no improvement within the past 25 years in the 20% 5-year survival rate for patients with OS lung metastasis. Disease relapse with pulmonary metastasis continue to be the major causes for death (1). Poor survival rates for patients who present with pulmonary metastasis highlights the necessity of new therapeutic strategies. Boosting the patient's own immune system is one such option. Our group has previously showed that IL-11Rα specific CAR-T cells resulted in regression of lung metastasis in IL-11Ra overexpressing osteosarcoma tumors (9).Though the CAR T cells exhibited anti-tumor efficacy, there is a risk of organ toxicity since the targeted antigen is expressed on normal cells i.e. liver and endothelial cells. NK cell mediated immunotherapy is emerging as a promising therapeutic strategy for cancer as NK cells exhibit antigen independent cytolytic activity so that normal cells can be spared.

Our group had previously shown that aerosol IL-2 given in combination with NK cell therapy significantly augmented the antitumor efficacy of NK cells for OS lung metastasis. Adding aerosolized IL-2 to NK cell therapy decreased the tumor burden in the lung dramatically and improved the overall survival of mice; however, this therapy failed to eliminate tumors completely from the lungs (46, 47). Some novel strategies are needed to improve the overall survival of patients with OS pulmonary metastasis. Our focus has been to further augment the therapeutic efficiency of NK cell therapy. The main goal of this thesis is to investigate the role of entinostat, an HDAC inhibitor, on the efficacy of NK cell-mediated immunotherapy using our human OS pulmonary metastasis mouse model.

**Entinostat sensitize OS cells to NK-cell mediated cytotoxicity by increasing ligands for activating NK cell receptors**

NKG2D is a primary activating receptor expressed by NK and T cells against tumor targets. NKG2D interacts with target cell ligands heterogeneously expressed on tumor cells including MHC-class 1 related genes MICA and MICB and UL16-binding proteins (ULBP-1 through 6). These cell ligands are upregulated in response to cellular stress, however tumors may escape NK cell recognition by either suppressing NKG2RD receptor expression or by down regulating their ligand expression (80, 81)**.**

Our group along with others showed that OS cells are susceptible to NK-cell mediated cytotoxicity due to the high expression of ligands for NK cell activating receptors (32, 45, 46). The aim of this study was to augment the efficacy of NK cell therapy against OS pulmonary metastasis by up-regulating NK cell ligands on the tumor cells. It is known that besides the immediate inhibitory effects of HDAC inhibitors on tumor growth, they also enhance recognition of tumor cells by immune cells (80). Our data showed that entinostat up-regulated expression of ligands specific for NK cell activating receptors (MIC A/B, ULBP1, ULBP2/5/6, and CD155) in OS cell lines (chapter 2, figure 1). Protein and mRNA levels of MICA and MICB were upregulated in a dose and time dependent manner consistent with our previously published results (58). Expression of up-regulated ligands on OS cells was stable for more than 24 h after drug removal from the culture (chapter 2, table 1); this provide an  evidence that there would be sufficient time for NK cells to recognize and kill tumor cells in our mouse model.

Our laboratory demonstrated in *vitro* that NK cell mediated cytotoxicity is directly correlated with the number of NKG2D ligands expressed on OS cells, e.g. KRIB cells were less sensitive to NK cell lysis than LM7 and CCH-OS-D cells due to their lower level of NKG2D ligands expression (46, 47). In this study we showed that up-regulation of NK cell ligands on OS cell lines by entinostat increased their susceptibility to NK cell-mediated cytotoxicity, and that the NK cell function was abrogated by NK cell receptor blockade (chapter 2, figure3, 4). Our results are in agreement with many other's published data. Burghuis et. al., demonstrated that HDAC inhibitors, including entinostat, increased the susceptibility of Ewing sarcoma cells for NKG2D-dependent cytotoxicity by NK cells (59). Schmudde et.al showed that the HDAC inhibitors SAHA, sodium butyrate, and entinostat treatment increased the susceptibility of prostate carcinoma and medulloblastoma cell lines for NK killing using the same mechanism (60). These results are also in accordance with published data concluding that HDAC inhibitors increase the susceptibility of tumor cells to NK cell-mediated cytotoxicity by increasing the expression of MICA/B (61-63). These finding suggest that sensitizing OS cells for NK cell killing by HDAC inhibitors may be an approach to improve the efficacy of NK cell immunotherapy.

**Effect of entinostat on NK cell viability, receptor expression, and cytotoxic function**

Although increased expression of NK cell ligands on OS cells with entinostat treatment resulted in enhanced tumor recognition and lysis, the adverse effects of the drug on NK cells might diminish this benefit. To evaluate this possibility, we examined the effect of entinostat on NK cell viability, receptor expression, and cytotoxic function. Our findings demonstrated that entinostat was not cytotoxic for NK cells and did not affect their viability within 48 hours of treatment (chapter 3, figure 5). Previous studies indicated that even low dose of entinostat (0.1 μM) increased NKG2D expression on freshly purified human NK cells (58). Here we showed that exposure to ≤ 2 μM of entinostat for 24 h resulted in no change in *ex vivo* expanded NK cell receptor expression (NKG2D, NKp30, NKp44, NKp46, and DNAM-1). However, after 48 hours, higher doses of the drug (≥ 0.5 μM) down-regulated NK cell receptors expression except for the DNAM-1 (chapter 3, figure 6). Based on these findings, the *in vivo* study was designed in which the administration of the drug and infusion of NK cells were at least 24 hours apart to avoid down-regulation of NK cell receptor expression.

Importantly, our data demonstrated that 24 hours pretreatment with entinostat did not affect the NK cell-mediated cytotoxicity against OS cells (chapter 3, Figure 7). Contrary to our results, Ogbomo et al., showed that VPA (a broad-spectrum class I- and IIa-specific HDACi) and SAHA (a pan-HDACi) suppressed NK cell lytic activity (77). However, they did not include entinostat in their study. The differences in the reported effect of HDAC inhibitors on NK cells may be

explained by the relative specificity of HDAC inhibitors for various HDAC isoforms and their ability to inhibit the function of a specific HDAC. Ogbomo et al., used freshly purified NK cells from blood buffy coats in their study. By contrast we used activated *ex vivo* expanded NK cells. Presently, the exact mechanism involved for the disparity between two studies is unclear. In conclusion our findings suggest that combining entinostat with NK cell therapy should not interfere with NK cell-mediated cytotoxicity.

**Entinostat's mechanism of action and its regulation of MICA/B expression in OS cells**

We proposed two mechanisms by which entinostat up-regulates MICA/B expression in OS cells (figure 27). As an HDAC inhibitor, entinostat promotes activation of gene transcription by inducing histone acetylation on chromatin. We demonstrated that entinostat may increase MICA and MICB gene transcription by acetylation of histone 4 linked to the MICA and MICB gene promoters; but not through enhancing acetylation of histone 3 (chapter 4, figure 9). This may be unique to OS cells as previous reports showed that treating colon carcinoma cells with entinostat resulted in up-regulation of MICA and MICB expression by acetylation of histones 3 linked to MICA and MICB gene promoters (58). The histone deacetylase inhibitor SAHA has also been shown to enhance MICA/B gene transcription by increasing MICA-associated histone acetylation (78).

The second mechanism we investigated for up-regulating MICA and MICB expression is the role of miRNAs i.e miR-20a, miR-93, and miR-106b (73). Our

data showed that these 3 miRNAs down-regulated MICA and MICB expression in OS cells (chapter 5, figure 10, 11). We further demonstrated that down-regulation of MICA and MICB expression by miR-20a, miR-93, and miR-106b is not controlled by MICA and MICB mRNA degradation. We are the first to show that entinostat leads to the up-regulation of MICA/B by down-regulating miR-20a, miR-93, and miR-106b in OS cells *in vitro* and *in vivo* (figure 12). Similarly, it has been demonstrated that SAHA upregulates the transcription of MICA/B by suppressing the MICA/B-targeting miRNAs miR-20a, miR-93 and miR-106b in hepatocellular carcinoma (78).

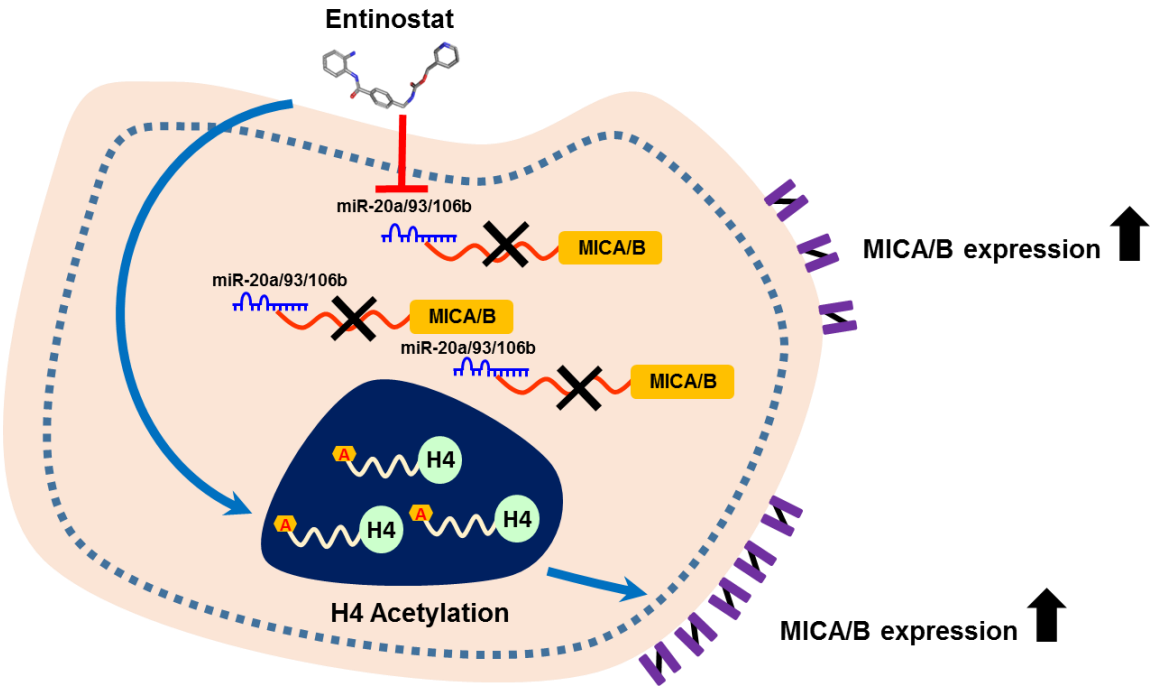



**Figure 27. Mechanisms by which entinostat increases MICA/B expression in OS cells.** Entinostat increases the accumulation of acetylated histone 4 in chromatin linked to both MICA and MICB genes and results in MICA and MICB gene up-regulation. Entinostat also increased MICA and MICB expression by down-regulating mir-20a, mir-93, and mir-106b expression in OS cells.

**NK cell therapy in combination with entinostat in mice with OS pulmonary metastasis**

Clinical data suggest that NK cells may have a role in OS prevention and prognosis. For example, study on children with OS showed that they had fewer number of peripheral NK cells compared to the control group. This suggests a role for NK cells in preventing OS (42). OS patients who had early lymphocyte recovery (including NK cells) after chemotherapy showed better prognosis than patients with late lymphocyte recovery (43). Further, OS patients treated with IL-2 in addition to chemotherapy had higher number of NK cells and the degree of the NK cell activity and the number of NK cells significantly correlated with the clinical outcome (82).

Having demonstrated that entinostat increases the susceptibility of OS cells to NK cell lysis *in vitro* (described in Chapter 2), we next determined whether entinostat in combination with NK cell infusion enhances the anti-tumor efficacy of NK cells in a nude mouse model with established OS pulmonary metastasis. Contrary to our *in vitro* findings, our results showed that combining oral administration of entinostat and NK cells infusion did not decrease the tumor burden in the lungs (chapter 6, figure 18).

Having shown that *ex vivo* expanded NK cells used for immunotherapy were fully activated and highly cytotoxic to LM7 cells (chapter 6 figure 15), it is unlikely that the ineffectiveness of NK cells to eliminate the tumor cells *in vivo* was due to their impaired function. Further, we demonstrated that the sub-therapeutic dose of entinostat (5 mg/kg) used was enough to significantly increase NKG2D ligand (MICA and MICB) expression on OS lung metastasis (chapter 6 figure 14, 22).

Taken together, these data indicate that entinostat had done its predicted role in our therapy.

The efficacy of immunotherapy for cancer is highly dependent on the effector to target cell ratio. Thus, for the second animal study we used genetically modified OS cell line LM7 expressing firefly luciferase to be able to monitor micrometastasis formation in the lungs by bioluminescent imaging and initiate therapy at the earliest time. We started the treatment when >75% of the mice developed micrometastasis, and we observed similar results as the first animal study (chapter 6, figure 20). This result indicates that the therapy's ineffectiveness was probably not secondary to high tumor Burden.

In order for NK cell therapy to be effective, the cells must reach the target organ (lung) and penetrate into the tumor. We showed that infused NK cells are able to traffic to the mouse lungs. However, these NK cells failed to infiltrate into the lung nodules to eliminate the tumor cells (chapter 6 figure 23, 24). This may explain why entinostat together with NK cells had no additive effect on tumor regression. In addition to this, the presence of immune suppressive cells and/or absence of activating and chemotactic factors in tumor microenvironment may have played a role in the lack of therapeutic effect by NK cells.

*Ex vivo* activated NK cells have a limited survival time and show low tumor penetration without cytokine support *in vivo* (83, 84). IL-2 promotes activation, proliferation and survival of NK cells, resulting in enhanced cytotoxic function for numerous different tumor cells (85). Most of the clinical trials of adoptive NK cell therapy use the subcutaneous administration of IL-2 to patients (31, 86, 87). Our

research group used aerosol IL-2 in combination with NK cells to increase their anti-tumor efficacy to OS lung metastasis and to avoid the significant side effects that accompany systemic IL-2 administration (46, 47). Aerosol IL-2 dramatically increased the number of infused NK cells in the metastatic pulmonary nodules resulting in increased tumor regression and improved overall survival of mice compared to those treated with NK cells alone. Another study also showed that NK cell immunotherapy decreased the tumor burden in the tibia, reduced bone damage, and raised the overall survival in NSG mice with OS lung metastasis only when IL-2 (10,000 IU IP injection /mouse) was added to the therapy (45). All these results suggest that adding IL-2 cytokine support to entinostat and NK cell combination therapy may be beneficial for the persistence in the lung, penetration into the tumor and enhanced cytotoxic function of infused NK cells *in vivo*. This is even more crucial in our mouse model in which the crosstalk between NK cells and T cells is absent due to the lack of T cells in nude mice. T cell interaction with other immune cells including NK cells enhance immune response against tumors. CD4+ T cells are known to be the main source for IL-2 production and tumor antigen-specific CD8+ T cells has been shown to provide stimulatory signals for NK cells in the tumor microenvironment setting (88, 89). In conclusion, although we demonstrated that entinostat increased the expression of NK cell ligands on OS lung metastasis, this may not have been enough to augment the efficacy of NK cell therapy due to the absence of factors (such as cytokines and chemokines) augmenting NK cell proliferation and survival in the tumor microenvironment.

Combining NK cell therapy with cytokines such as IL-2 or IL-21 may provide the needed additional support.

It is well known that tumor cells can induce immune escape mechanisms (90). Many tumors down-regulate or internalized their NKG2D ligands (91) or reduce MICA and MICB surface levels by ligand proteolytic shedding from the cell surface (92-94). Reduced expression of MICA and MICB on the tumor cell surface and increased levels of their soluble forms in the tumor microenvironment and serum can impair NKG2D-mediated cytotoxicity of NK cells. High levels of soluble MICA (sMICA) in OS patient's serum has indeed been detected (95). In another study serum and tumor biopsies from OS patients before radiotherapy and chemotherapy showed high expression levels of MICA (96). Very high serum levels of sMICA were detected in one third of the patients but not in healthy controls and elevated sMICA levels were associated with the disease progression and rate of metastasis. Therefore, in our mouse model the proteolytic shedding of MICA may also have interfered with NK cell cytotoxicity. Future studies investigating this aspect might are warranted.

PD-L1 up-regulation on tumor cells contributes to tumor immune-escape (97, 98). Interaction between the PD-1 receptor, expressed on immune cells, and its ligand PD-L1, expressed on tumor cells, leads to immune cell anergy, and apoptosis and allows tumor cells to escape from immune-mediated cytotoxicity. Blockade of the PD-1/PD-L1 interaction in patients with solid tumors lead to improved clinical outcome (99, 100). PDL-1 has been reported to be expressed on OS cell lines as well as OS tumor samples (101-104). Blocking PD-1/PD-L1 axis

in K7M2 mice with OS pulmonary metastasis resulted in enhanced T cell cytotoxicity, tumor regression and improved survival. However the impact of PD-L1 blockade on NK cell function has not been studied in this mice model (103). Our *in vitro* data demonstrated that entinostat upregulated PD-L1 expression on OS cell lines in a dose dependent manner (figure 25); approximately 6-8% of *ex vivo* expanded NK cells expressed the inhibitory receptor PD-1 (figure 26). Hence we proposed that up-regulation of PD-L1 on metastatic OS by entinostat may compromise tumor reactive PD-$1^{+}$ NK cell function leading to reduced anti-tumor effect. However, we have not determined the level of PD-L1 on OS lung metastasis following the infusion of NK cells *in vivo*.

Malignant cells can also interfere with anti-tumor efficacy by producing immune suppressive factors such as TGF-β, lL-10, and Macrophage Migration Inhibitory Factor (MIF), or by recruiting immunosuppressive leukocytes such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells into the tumor microenvironment (90, 105). TGF-β1 secreted by tumor cells can down-regulate NKG2D and NKp30. Overexpression of MIF on ovarian cancers is associated with the down-regulation of NKG2D on NK cells (106-109). Investigating the effect of TGF- β and MIF on NK cell functionality in our *in vivo* studies might provide insight for the reduced anti-tumor effect in mice.

## Conclusions

The immune system is a critical regulator for controlling cancer growth. Adoptive immunotherapy is a promising approach to combat cancer. However, cellular immunotherapy including NK cell therapy seems to be more challenging in patients with solid tumors. In this study we demonstrated that HDAC inhibitor, entinostat, augmented NK cell-mediated cytotoxicity for several different OS cell lines by up-regulating the ligands specific for NK cell activating receptors. Up to 2 μM entinostat for 24 hours did not change NK cell viability, or the activating receptor expression and function. We also demonstrated two possible mechanisms by which entinostat increases NKG2D ligands (MICA and MICB) in OS cells, first by increasing histone 4 acetylation on the MICA and MICB gene promoters, and second by down-regulating mir-20a, mir-93, and mir-106b expression. We did not observe significant differences in the tumor burden in mice treated with entinostat plus NK cells compared with entinostat or NK cells alone. Our data suggest that the reason for the lack of a therapeutic effect is secondary to the failure of the NK cells to traffic into the tumor nodules. Although, NK cells migrated into the lung, we only observed NK cells at the periphery of the tumor and not in the center.

Entinostat treatment also resulted in up-regulation of immune inhibitory molecules PD-L1 on OS cell lines. Thus, blockade of PD/PDL1 axis with PD-L1 monoclonal antibodies may improve the anti-tumor effect of NK cells against the OS lung metastasis. More studies are needed to reveal the mechanism of resistance.

## FUTURE DIRECTIONS

### Enhancing NK cell infiltration and retention in osteosarcoma lung metastasis by aerosol IL-2 or IL-21 administration

Our data demonstrated that treating OS cells with entinostat up-regulated the expression of NK cell activating ligands on OS cells and increased NK cell-mediated cytotoxicity *in vitro*. However, while the oral administration of entinostat also resulted in increased ligand expression *in vivo*, combination therapy did not augment the efficacy of NK cell therapy against OS lung metastasis. One of the reasons could be the inability of infused NK cells to infiltrate into the OS lung nodules. We observed the infused NK cells only at the periphery of the tumors. Since NK cells are highly dependent on cytokine milieu support like IL-2 and IL-21 for their function, proliferation, and retention in the tumor microenvironment, we propose that adding a stimulatory cytokine to the treatment may increase the efficacy of NK cell therapy with or without entinostat. This would create a dual effect, i.e. increases the susceptibility of OS cells to NK cell-mediated killing by enhancing the expression of NK cell ligands and increasing NK cell efficacy by increasing the penetration of the NK cells dipper into the tumor tissue.

To avoid the side effects of systemic IL-2 administration and to activate the injected NK cells in the lung specifically, we propose using aerosol IL-2 delivery. To assess this, nude mice would be injected i.v. with $2 \times 10^6$ LM7 cells to establish micrometastasis in the lungs. A similar treatment schema that we used in our study will be followed with the exception of adding of aerosol IL-2 administration (mice

groups: aerosol IL-2; NK cells+ IL-2; entinostat+ IL-2; or NK cells+ entinostat+ IL-2). For example, recombinant human IL-2 will be given at 2000 U/mouse three times a week for five weeks (Table 3). The safety of aerosol IL-2 has been reported both in clinical trials and in our mouse model as well as in immunocompetent BALB/C mouse (46, 110, 111). We have previously demonstrated that no acute or chronic inflammation, toxicity, elevated liver enzymes or abnormal CBC was seen following aerosol IL-2 administration, supporting the concept of using organ-specific delivery as a way to treat lung metastasis (46, 47).

After 5 weeks of treatment, the number of visible nodules would be quantified and the weight of lungs will be compared with the control group in order to evaluate the therapeutic effect of combination therapy. Paraffin-embedded OS lung metastasis tissues will be stained for the NK cell marker NKp46 to assess the number of infiltrated NK cells in the lung nodules. We anticipated that aerosol IL-2 will result in increased NK cells inside the tumor nodules. The correlation between the number of NK cells in the lung nodules and the tumor apoptosis (using TUNEL staining) will be assessed. We anticipated that increased NK cell content will correlate with increased apoptosis. Survival will also be evaluated. We predict that aerosol IL-2 therapy will enhance infiltration, and retention of NK cells in tumors resulting in decreased tumor burden in the lungs and improved survival.

| Groups | | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|---|---|
| Control | | DMSO+ Aerosol IL-2 | | | DMSO+ Aerosol IL-2 | | DMSO+ Aerosol IL-2 | |
| Entinostat | | Entinostat+ Aerosol IL-2 | | | Entinostat+ Aerosol IL-2 | | Entinostat+ Aerosol IL-2 | |
| NK cells | | DMSO+ Aerosol IL-2 | NK cells | | DMSO+ Aerosol IL-2 | NK cells | DMSO+ Aerosol IL-2 | |
| NK cells+ Entinostat | | Entinostat+ Aerosol IL-2 | NK cells | | Entinostat+ Aerosol IL-2 | NK cells | Entinostat+ Aerosol IL-2 | |

**Table 3. Experimental plan for combination therapy with entnostat, NK cells and aerosol IL-2 in mice with OS lung metastasis.** Mice will be treated with entinostat at a concentration of 5 mg/kg of weight by oral/gavage three times a week (Monday, Thursday, and Saturday) for five weeks. Recombinant human IL-2 will be given by aerosol delivery at 2000 U/ mouse three times (Monday, Thursday, and Saturday) for five weeks. 50x $10^6$ NK cells will be injected via tail vein two times in a week (Tuesday, and Friday) for 5 weeks.

Another alternative for increasing NK cell infiltration into the OS lung metastasis is the use of Interleukin‑21 (IL-21). This cytokine is mostly produced by NKT, $CD4^+$ T, and Th17 cells. IL-21 regulates T, B, and NK cells function (112). The antitumor activity of IL-21 has been shown in various preclinical investigations (113-115). In clinical trials, the safety and efficacy of IL-21 has been demonstrated for metastatic melanoma and renal cell carcinoma (116-118). Furthermore, it has been shown that IL-21 stimulated NK- and $CD8^+$ T cell-mediated cytotoxicity against mantle cell lymphoma, and renal cell carcinoma *in vivo* (119, 120). In addition, tumor regression in mice bearing fibrosarcoma or melanoma after treatment with IL-21 was dependent on NK cell-mediated cytotoxicity rather than T

cell cytotoxicity (121). These findings suggest that adding IL-21 to our treatment may enhance the efficacy of NK cell therapy with or without entinostat. In the latter study, injection of a plasmid DNA encoding murine IL-21 resulted in increased and constant expression of IL-21 in the blood (121). Murine IL-21 plasmid can be administered through tail vein injections and the maximum tolerable DNA dose would be determined in our mouse model as previously described (122, 123). The volume of the DNA solution and number of injections will be optimized. Based on the method used in the previous studies, various amounts of plasmid in 1-3 ml of saline within 5-7 second will be administered (121-123). More than one injection per mouse may be needed to gain the maximum plasmid DNA expression in hepatocytes.

After optimizing the conditions for plasmid administration, we would follow the same treatment schema that we used for aerosol IL-2 but would substitute systemic IL-21 plasmid DNA. After 5 weeks of treatment, mice will be sacrificed and lungs will be weighed and examined for the number of nodules. The number of infiltrated NK cells and its correlation with the tumor cell apoptosis and regression will be evaluated as well. The presence of organ cytotoxicity and elevated liver enzyme levels due to IL-21 administration will also be assessed. We expect that administration of IL-21 would result in increased number of NK cells in the OS lung tumors along with the enhanced NK cell anti-tumor effect as demonstrated by increased tumor cell apoptosis, decreased numbers and size of lung metastasis and an improved survival rate.

The limitations of these studies is our nude mouse model which does not exactly mimic the human clinical setting due to absence of T cells. T cell crosstalk with other immune cells including NK cells has been shown to enhance the immune response against tumors. For example, T cells secrete cytokines such as IL-2 and IL-21 that play an important role in NK cell activation. IL-2 activates both cytotoxic T and immunosuppressive regulatory T cells (124). Although, activation of regulatory T cells is not a concern in our nude mouse model, this can be a limitation for IL-2 administration in a clinical setting.

Crosstalk between macrophages and NK cells may also play a role in NK cell-mediated tumor regression. Tumor-associated macrophages (TAMs) are known to be the most dominant immune cells in the tumor microenvironment (125). M1 and M2 macrophages are two subtypes of macrophage with completely different functions in the tumor setting. In the initial phase of tumor development, the tumor microenvironment induces the polarization of TAMs toward the M1 phenotype while the M2 phenotype is dominant during tumor progression (126). By producing Th1-type cytokines, e.g. IL-12 and TNFα, M1-polarized macrophages induce anti-tumor responses. In the Th1/M1-polarizing context, interaction between different immune cells like macrophages, DCs, and neutrophils results in NK cells activation and enhanced anti-tumor effect. Alternatively, activated NK cells may further promote activation of other immune cells such as macrophages, T cells, and DCs and enhance Th1/M1 polarization (19, 127-131). In contrast, M2-polarized TAMs enhance tumor progression by producing immunosuppressive cytokines (TGF-ß and IL-10) and promote

angiogenesis, stroma formation, tissue reconstruction, and metastasis. High numbers of M2-polarized macrophages in the tumor microenvironment may correlate with the tumor progression and poor prognosis (132) and may also contribute to the inability of NK cells to penetrate into the tumor due to the stoma formation. Since nude mice do have macrophages this may have contributed to the inability of NK cells to migrate into the lung tumors.

In our *in vivo* studies, administration of aerosol IL-2 may activate mouse NK cells in the lung nodules since mouse NK cells can be activated by human IL-2 (46, 133). Activated NK cells may produce cytokines and immune stimulatory factors (INF- γ, TNFα, GM-CSF, and MIP-1) that induce M1 polarization. To investigate this possibility, IHC staining for M1 and M2 TAMs on OS lung metastasis samples may be performed using their markers CD86 and CD163, respectively. Flow cytometry of dissociated tumor cells stained with antibodies specific for M1 and M2 would also be performed. It would be important to evaluate if entinostat has any modulatory effect on macrophage and the ratio of M1 and M2 macrophages in our mouse model. Interestingly, higher number of macrophages in the pretreated biopsies of patients with OS correlated with longer overall survival and fewer metastasis (134). These findings suggest a rationale for using macrophage activating agents such as L-MTP-PE in patients with OS. Combining L-MTP-PE with the standard chemotherapy resulted in a 29% decrease in mortality rate in newly diagnosed OS (15) and significantly increased the disease-free survival rate in relapsed OS patients (14). Therefore, using L-MTP-PE together

with other immune therapies which increases macrophage infiltration into the lung metastasis may be beneficial.

**Investigating the interference of soluble forms of MICA and MICB with the NK cell therapy in mice with OS lung metastasis**

Stress-inducible MICA and MICB ligands are heterogeneously expressed on tumor cells. Many tumors escape from the immune system by proteolytic cleavage of the ligands and releasing the soluble forms of MICA, MICB (sMICA and sMICB) or other NKG2D ligands (135-138). The process of ligand shedding results in decreased expression of MICA and MICB on the tumor cell surface and increased soluble forms in the serum and microenvironment, which in turn can impair NKG2D-mediated cytotoxicity of NK cells and cytotoxic T lymphocytes. Recently, the presence of soluble forms of NKG2D ligands has been demonstrated as a prognostic factor and diagnostic biomarker for cancer (139, 140). In fact, the mechanisms involved in the release of these ligands from the cell membrane are considered as potential targets to be blocked with the goal of increasing the efficacy of antitumor immunity.

Elevated levels of MICA in the serum of OS patients are correlated with the stage of the disease and rate of metastasis. Further, MICA expression on OS tissues was adversely correlated with the stage of the disease (95, 96). Previous studies demonstrated that Matrix metalloproteinase 9 (MMP9) may be responsible for MICA shedding from the OS cell surface (141) and other tumors (142). Down-

regulation of MMP9 resulted in reduced MICA cleavage from OS cells (143, 144). MMPs are over-expressed in many tumor cells and clinical trials are underway using MMP-inhibitors in cancers. MMPs consist of 24 human zinc-binding endopeptidases that play different roles in tumor cell growth, survival, invasion, and migration (145). Proteolytic cleavage by MMPs is known to be the main mechanism involved in the release of MICA, MICB, and ULBP2 from the cell membrane (146).

Thus, it would be interesting to investigate the role of sMICA and sMICB in the NK cell therapy in our mouse model. Valproic acid has been shown to up-regulate MICA and MICB expression on OS cells and reduce the sMCA and sMICB shedding due to the down-regulation of MMP9 expression (143). However, UCI-10, an ovarian cancer cell line, released more sMICA and sMICB after being treated with HDAC inhibitor trichostatin A both *in vitro* and *in vivo* (94). The effect of entinostat on sMICA and sMICB secretion is unknown at present. To investigate this, sMICA and sMICB levels will be measured in control and entinostat-treated OS cell culture media by ELISA. Next, NK cells will be mixed with the culture media of control and entinostat-treated OS cell lines. The cytotoxicity of these two groups of NK cells against OS cells would be assessed by calcein release assay. Since the effect of entinostat on MICA and MICB shedding from OS cells is unknown, the results of these experiments cannot be predicted.

In order to determine whether shedding of MICA and MICB can be reduced by MMP9 inhibitor and to investigate how this process will be affected by entinostat, we will treat OS cells with various doses of MMP9 inhibitor with or

without entinostat treatment. As other studies have shown, we expect that the inhibitor would reduce the shedding of sMICA and sMICB (94, 143, 144). The effect of the MMP9 inhibitor on the NK cell-mediated tumor cytotoxicity against OS cells would be assessed. NK cells will be mixed with the culture media from untreated or treated OS cells with MMP9 inhibitor and then their cytotoxicity against OS cells would be evaluated using calcein release assay. We expect that media from OS cells treated with MMP9 inhibitor would have less ability to block the NK cell-mediated cytotoxicity due the lower levels of MICA and MICB in the media.

To assess the *in vivo* effect, nude mice bearing OS lung metastasis will be treated with a sub-therapeutic dose of entinostat with or without the MMP9 inhibitor. Serum levels of sMICA and sMICB will be measured in each group. Lung nodules will be stained for MICA and MICB expression. If we find enhanced MICA and MICB expression on the lung metastasis and lower levels of sMICA and sMICB in the serum from mice treated with both entinostat and MMP9 inhibitor, then we will use the combination of MMP9 inhibitor and entinostat to investigate whether the inhibitor can increase penetration of NK cells into the tumor and the efficacy of NK cells in mice established with OS pulmonary metastasis.

**The effect of anti-PD-L1 antibody on the efficacy of combination therapy of NK cells and entinostat in mice with OS lung metastasis.**

PD-L1 up-regulation on tumor cells inhibits the anti-tumor activity of immune cells by triggering inhibitory signals into the immune cells following PD-L1/PD-1 interaction (11). PDL-1 has been shown to be expressed on OS cell lines as well as OS tumor samples (101-104). *In vitro*, we demonstrated that entinostat enhanced PD-L1 expression on OS cell lines in a dose dependent manner. However, the effect of entinostat on PD-L1 expression on OS lung metastasis is unknown at this time. This effect can be investigated by treating nude mice bearing OS lung metastasis with 5 mg/kg entinostat or DMSO 3 times a week for 2 weeks. The level of PD-L1 expression on the lung tumors would be determined using IHC staining and flow cytometry of dissociated tumor cells stained for PD-L1. If our results show that entinostat up-regulates PD-L1 expression on OS lung metastasis, we will add anti-PD-L1 to our combination therapy to block the PD-1/PD-L1 axis. Using PD-L1 antibody in Balb/cJ mice with OS pulmonary metastasis resulted in enhanced T cell cytotoxicity, tumor regression and improved survival. However the impact of PD-L1 blockade on NK cell function has not been studied in this mouse model (103).

A similar treatment schema as described in the results section will be used with the addition of anti-PD-L1 (mice groups: control; NK cells; entinostat; PD-L1 mAb; NK cells+ entinostat; NK cells+ PD-L1 mAb; entinostat+ PD-L1 mAb, or NK cells+ entinostat+ PD-L1 mAb). 200 µg PD-L1 antibody (10F.9G2, BioLegend) would be administered every three days for five weeks (147). At the end of

treatment, mice will be sacrificed and lungs will be examined for the number and size of the nodules. The number of infiltrated NK cells in the lung tumors and its correlation with the tumor regression will be also determined in each group. PD-L1 expression on lung tumors will be determined by IHC staining. Infiltrated NK cells into the lung tumors will be isolated and analyzed by flow cytometry to determine whether the expression of PD-1 is different. If the *in vivo* results show that administration of PD-L1 antibody enhances the efficacy of NK cell therapy, then it would be interesting to create and inject PD-L1 shRNA knockdown LM7 cells into nude mouse to establish OS metastasis and to investigate the efficacy of NK cell combination therapy with entinostat in the absence of PD-L1 expression on tumor cells.

# Chapter 8

# Materials and Methods

**Reagents and antibodies**

Entinostat was purchased from Sigma-Aldrich (St. Louis, MO). For *in vitro* experiments, entinostat was dissolved in absolute ethanol and then diluted in Dulbecco's modified Eagle's medium (DMEM) for working solutions. For *in vivo* experiments, entinostat was dissolved in DMSO. Anti-human acetyl-histone3, anti-human acetyl-histone4, anti- human acetyl-histone H3 antibody, and anti- human acetyl-histone H4 antibody were purchased from Millipore (Temecula, CA). Anti-human MICA/B was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell lines**

The metastatic human LM7 OS cell line used in our study was derived from the parental SAOS-2 cell line by re-cycling the cells 7 times through the lungs of nude mice. KRIB, CCH-OS-D, and CCH-OS-O OS cell lines were kindly provided by Dr. Dennis Hughes from MD Anderson Cancer Center. CCH-OS-D and CCH-OS-O are primary OS cell lines derived from patient samples at the Children's Cancer Hospital at MD Anderson Cancer Center. Cell lines were cultured in DMEM supplemented with 1% streptomycin/penicillin, 1 mmol/L nonessential amino acids, 2 mM L-glutamine, 1 mmol/L sodium pyruvate, and 10% fetal bovine serum (Intergen, Purchase, NJ). All cells were incubated at 37°C with 5% CO2. Cells were split no more than 4 times before they were injected intravenously (i.v.), and they were free of mycoplasma.

**Animals**

Four- to six-week-old nu/nu mice were purchased from the National Cancer Institute Mouse Repository, Frederick, MD, and kept in pathogen-free conditions in a laminar air flow room as approved by the American Association for Accreditation of Laboratory Animal Care. Experiments were conducted according to the animal protocols approved by the Institutional Animal Care and Use Committee (IACUC).

**Human NK cell isolation and expansion**

Human NK cells were isolated from healthy donors' buffy coats. Then they were expanded *in vitro* for 4 weeks using genetically engineered K562 and recombinant human IL-2, as previously described (36). Briefly, NK cells were purified from blood buffy coats using RosetteSep Human NK Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC) and buoyant density centrifugation on Ficoll-Paque (GE Healthcare Life Sciences, Little Chalfont, UK). Isolated NK cells were seeded with irradiated K562 (100 cGy) at a ratio of 1:2 in RPMI 1640 medium (Cellgro/Mediatech, Manassas, VA). RPMI was supplemented with 10% fetal bovine serum (Intergen, Wellington, New Zealand), 2 mmol/L glutamine , 1 mmol/L sodium pyruvate, and 50 IU/ml recombinant human IL-2. Every 3 days, half of the NK cells medium was replaced with fresh RPMI and then fresh IL-2 was added to the entire media. At the end of each week, NK cells were re-stimulated with irradiated K562 at the ratio of 1:1 and re-suspended in fresh RPMI plus 50 IU/ml recombinant human IL-2. Four weeks after expansion, NK cells

were frozen in freezing medium (FBS+ 10% dimethyl sulphoxide, DMSO) at a concentration of $5x10^7$ cells/ vial and kept at -80$^o$C for further use. By end of 4-weeks of expansion, NK cell purity was ≥98%. Before NK cells were infused into mice, their viability and receptor expression were evaluated with use of Vi-CELL and flow cytometry, respectively. Furthermore, to confirm that expanded NK cells can recognize and lyse LM7 cells, their cytotoxicity against LM7 cells was evaluated by calcein release assay.

**Flow cytometry analysis**

NK cell phenotypes were analyzed weekly with flow cytometry using murine anti-human CD16-PE, CD3-PE, NKG2D-PE, and CD56-APC antibodies (BD Pharmingen, San Jose, CA). Murine anti-human HLA/ABC-PE, MICA/B-PE (BD Pharmingen), ULBP2/5/6-PE, ULBP3-PE, and ULBP-PE (R&D Systems) antibodies were used to determine HLA and NKG2D ligand (NKG2DL) expression. Anti-human Nectin-2/CD112 and anti-human CD155/PVR antibodies from R&D Systems were also used to detect NK cell ligands on OS cells. Flow cytometry was performed on a FACSCalibur cytometer (BD Biosciences) and data were analyzed with use of FlowJo software (Tree Star, Inc., Ashland, OR).

**Western blot analysis**

LM7 OS cells were treated with 0.5, 1.0, or 2 μM entinostat for 48 h and collected and lysed with RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Santa Cruz Biotechnology, Inc.). Total protein

concentration in each sample was analyzed by a Bicinchoninic acid (BCA) assay kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Protein samples were boiled in SDS loading buffer for 5 minutes before loading onto 10% of SDS-poly acrylamide gels (SDS-PAGE). Samples were electrophoresed at 90 V for 2 ½ h and transferred to nitrocellulose membranes at 85 V for an hour. Samples were then blocked in 5% milk or BSA at room temperature for 1 hour. Expression of each individual protein was examined by overnight incubation with primary antibody at 1:1000 ratio at $4^0$C against MICA/B, acetyl-histone3, or acetyl-histone4 and visualization by horseradish peroxidase (HRP) conjugated secondary antibody. Signal was detected after incubation with enhanced ECL reagent (GE healthcare life sciences). Beta actin was used as loading control.

**Real-time polymerase chain reaction**

LM7 OS cells were treated with 0.5, 1.0, or 2 μM entinostat for 48 h. Total RNA was isolated and purified from treated and untreated LM7 with use of Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). Reverse transcription was performed by using the ReverseTranscription System with oligo-dT primer (Promega Corporation, Madison, WI) according to the manufacturer's instructions. mRNA levels of MICA and MICB were measured by real-time polymerase chain reaction by using iQ SYBR Green Supermix (Bio-Rad Laboratories). The PCR reaction mixture of 25 μL was prepared by using 100 ng of reverse-transcribed total RNA as template, 50 nM each forward and reverse primers, and 12.5 μL of SYBR green buffer (Bio-Rad Laboratories Inc., Hercules, California). Specific

primers for MICA and MICB were as follows: MICA, 5'-AGGGTTTCTTGCTGAGGTACA-3' (forward) and 5'-GGTCTCTCTGTCCCATGTCTTA-3' (reverse); MICB, 5'-TCTTCGTTACAACCTCATGGTG-3' (forward), and

5'-TCCCAGGTCTTAGCTCCCAG-3' (reverse). Cycling conditions were $95^0$C for 3 min, 45 cycles of 59 °C annealing for 30 s, 95 °C for 30 s, and 60 °C for 1 min.

For the miRNA assay, total RNA was isolated and purified from cultured cells with use of Trizol reagent. Reverse transcription was performed by using a TagMan miRNA reverse transcription kit according to the manufacturer's instructions. For each 15-µL RT reaction, 7 µL RT master mix, 5 µL total RNA (5 ng), and 3 µL of RT primer were combined. The resulting cDNA was subjected to PCR amplification with use of specific primers for Mir-20a, Mir-93, or Mir106b.

**Cytotoxicity assay**

NK cell cytotoxicity was measured by using a calcein release assay as described previously (58). Briefly, 1 x $10^6$ target cells were resuspended in 1 µM calcein-AM diluted in DMED and incubated at 37ºC for 1 h. Cells were washed with RPMI media twice and co-cultured with *ex vivo* expanded NK cells in 96-well U-bottom plates in a total volume of 200 µL in triplicate and at a ratio of 0.3125:1-1:10 (effector cell: target cell). Plates then were incubated at 37ºC for 4 h. Calcein-labeled target cells and calcein-labeled target cells treated with 2% Tween 20 were considered as spontaneous release and maximum release, respectively. A total of

100 µL of supernatant was transferred to a 96-well flat bottom plate. The plate was read at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Percent specific lysis was calculated according to the formula [(test release − spontaneous release) / (maximum release − spontaneous release)] × 100.

**Chromatin Immunoprecipitation (CHIP)**

LM7 cells were treated with 2 µM entinostat for 48 h. Chromatin immunoprecipitation was performed by using a CHIP assay kit (Millipore, Temecula, CA) according to the manufacturer's instructions. Briefly, cell lysates were treated with 1% formaldehyde to crosslink protein and DNA. Lysates were sonicated to shear chromatin to 200-1000 bp in length and immune-precipitated by antibodies specific to acetyl-histone H3, acetyl-histone H4, histone H3, histone H4, or respective isotype control IgG antibodies. MICA and MICB promoter regions were amplified by quantitative real-time PCR using primers specific to MICA or MICB. A 423-bp segment located at -439 upstream of the translation initiation site of the MICA (1), as well as a 233-bp of MICA (2) located at –215 to +18 of the gene were amplified by using the following primers: MICA (1) forward 5′-GAAGGAACAAGCCAGTG-3′, reverse 5′-GCCAGAAGCAGGAAGACC-3′; MICA (2) forward 5′-TTA GGC TGC GCT CCC GCG TGC TCC-3′, reverse 5′-CTC AGC GGC TCA AGC AGT GGC CGG-3′. Likewise, a 220-bp fragment of the MICB (1) promoter covering bases from –239 to –20 as well as a 678-bp located at MICB (2) gene promoter were amplified by using the following primers: MICB (1) forward 5′-GTT TGG AGC TGT ACT CTC AGC TAC-3′, reverse 5′-CCC GCT CAG CGA

CCG CTT ATC CAG-3′; MICB (2) forward 5′ GCGACAGGGTCCAGGTCGTGCTC-3′, reverse 5′-CCCTACGTCGCCACCTTC TCAGCT-3′. After normalizing the readings to inputs, the results were presented as a ratio of acetylated histone (H3 or H4) to total histone (H3 or H4).

**miRNA mimics transfection.**

miRNA mimics and the negative control of miRNA mimics were transfected into OS cells lines using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA). A total of $2 \times 10^5$ OS cells/well were seeded in a 24-well plate and transfected with the miRNA mimics at a final concentration of 5 pmol. Cells were collected at 24 and 48 hours and analyzed by a flow cytometry assay. Total RNAs were isolated and purified from cells by using Trizol reagent and subjected to RT-PCR and then quantitative real-time PCR.

**Determination of sub-therapeutic dose of entinostat for the *in vivo* study**

Female nu/nu mice (aged 4-6 weeks) were injected intravenously with $2 \times 10^6$ LM7 cells suspended in 0.2 mL of PBS solution. Visible nodule formation was confirmed 8 weeks later after euthanization. Mice then were treated with 2.5, 5, or 10 mg/kg entinostat 3 times a week for 2 weeks. At the end of treatment nodules were removed from the lungs and total mRNAs were extracted using Trizol reagent. MICA and MICB mRNA levels were evaluated by quantitative real-time PCR.

**Animal model**

Four- to six-week-old nu/nu mice were injected intravenously with 2× $10^6$ LM7 cells suspended in 0.2 mL of PBS solution. About 5-6 weeks later, a few mice were euthanized to evaluate lung micrometastasis formation. Thereafter, treatment was started in the following four mouse treatment groups: 1) DMSO; 2) entinostat; 3) NK cells+ DMSO; and 4) NK cells+ entinostat. Mice received 5 mg/kg of entinostat or 200 µL of DMSO (control) by oral gavage three times a week for 5 weeks. A total of 50× $10^6$ NK cells were injected via the tail vein twice a week for 5 weeks. A total of 12-16 mice were included in each mouse group. Mice were euthanized after 5 weeks of treatment. The lungs were removed, and the visible lung metastases were counted and measured. Fresh-frozen samples from the resected lungs were prepared for further use.

In the case of using luciferase-expressing LM7 cells, nude mice were injected via tail vein with 2 × $10^6$ LM7-Luc cells (a gift from Dr. Gottschalk from Baylor College of Medicine). Mice were monitored every week for micrometastasis formation in the lung by bioluminescent imaging. When micrometastases were observed in 75% of the mice, the mice were evenly distributed into four groups. Treatment was initiated with DMSO, entinostat, NK cells+ DMSO, or NK cells+ entinostat for 5 weeks. Mice received 5 mg/kg of entinostat or 200 µL of DMSO (control) by oral gavage 3 times a week for 5 weeks. A total of 50× $10^6$ NK cells were injected via the tail vein twice a week for 5 weeks. A total of 11-12 mice were included in each mouse group. Before treatment and at the end of treatment the

tumor burden was evaluated by bioluminescent imaging using the IVIS spectrum system (Xenogen). Mice were kept for the survival assay.

**Immunohistochemistry**

Paraffin-embedded lung tissues were used for analysis. After deparaffinization and rehydration of slides, antigen retrieval was performed by using sodium citrate. To block exogenous peroxidase, slides were incubated with 3% H2O2 for 12 minutes followed by PBS supplemented with 4% fish gelatin in PBS. Antibodies specific to MICA/B (abcam, US) was applied and left overnight at 4°C followed by incubation with horse radish peroxidase-labeled secondary antibodies for 2 hours at room temperature. Slides were then developed with 3, 3′-diaminobenzidine (DAB) as a substrate, and counterstaining was done with hematoxylin. Negative controls were prepared in the same manner as the samples except that they were treated with primary antibodies.

**Immunofluorescence**

To quantify the presence of infused NK cell in the mouse lung, 50 x $10^6$ CM-Dil labeled NK cells were injected via the tail vein. At 50 min and at 24 h after NK cell injection, the mice were euthanized and the lungs removed. The frozen sections of the lung tissues were stained for the nucleus using Hoechst 33342 nucleic acid stain (Molecular Probes) at 1:50,000 dilution in PBS. Slides were examined under a fluorescence microscope. Lung tissues from mice that did not receive CM-Dil labeled NK cells were used as the controls.

Frozen sections from our *in vivo* experiment were analyzed by immunofluorescence staining for NK cells. Slides were fixed with 4% formaldehyde for 10 minutes. Slides then were incubated with blocking solution (4% fish gelatin in PBS) for 20 minutes at RT. Goat anti-human NKp46 antibody (10 µg/ ml) was added (R&D System, US) and slides were incubated at 4$^{o}$ C overnight. After washing with PBS, slides were incubated with anti-goat secondary antibody for 1 hour at RT. After washing with PBS, nucleus were stained with use of Hoechst 33342 nucleic acid stain. Slides then were mounted and examined with the use of a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY)

**Statistical analysis**

The unpaired student *t-tes*t was used for statistical comparisons of groups. Survival studies were analyzed with use of a Mann-Whitney rank-sum test. P values of less than 0.05 were considered statistically significant.

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