Effect of entinostat on NK cell-mediated cytotoxicity against osteosarcoma cells and osteosarcoma lung metastasis

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ABSTRACT

There is a crucial need for a new therapeutic approach for osteosarcoma (OS) lung metastasis since this disease remains the main cause of mortality in OS. We previously demonstrated that natural killer (NK) cell therapy has minimal efficacy against OS metastasis. This study determined whether the histone deacetylase inhibitor entinostat could immunosensitize OS cells to NK cell lysis and increases the efficacy of NK cell therapy for OS lung metastasis. Entinostat upregulated ligands for NK cell-activating receptors (major histocompatibility complex [MHC] class I polypeptide-related chain A [MICA] and B [MICB]; UL16 binding proteins 1, 2, 5, and 6; and CD155) on OS cells both in vitro and in vivo and led to more susceptibility to NK cell-mediated cytotoxicity in vitro. Importantly, entinostat did not change NK cell viability, receptor expression, or function within the 24-h treatment. We also demonstrated two potential mechanisms by which entinostat enhanced expression of MICA and MICB on OS cells. Although entinostat upregulated ligands for the NK cell activating receptor on OS lung metastasis, it failed to augment the efficacy of NK cell therapy in our nude mouse human OS lung metastasis model. This can be partly explained by our finding that although the infused NK cells were active and functional and could penetrate into the lungs, they failed to infiltrate into the lung nodules. These challenges regarding cellular immunotherapy against solid tumors may be overcome by combination therapy, such as adding a NK cell-activating cytokine (IL-2 or IL-21).

Introduction

Osteosarcoma (OS) is a primary bone tumor that metastasizes primarily to the lung. Although remarkable improvements have been made in the prognosis of patients with primary OS over the past 30 y, patients with local relapse or metastasis still experience significantly lower survival rates. Current treatments, including surgery and combinational chemotherapy, have enhanced the 5-y survival rate of patients with nonmetastatic OS to 70%; however, for those with pulmonary metastasis, the 5-y survival rate is less than 20%. Therefore, it is imperative that a new therapeutic approach be developed for patients with OS lung metastases.

Natural killer (NK) cells are a subset of lymphocytes and part of the innate immune system that play a critical role against virus-infected and tumor cells. In contrast to T cells, NK cells do not require prior sensitization to antigens to recognize target cells. Indeed, they become activated by the signals transduced through a group of activating and inhibitory cell surface receptors. When the activating signals exceed the inhibitory ones, NK cells become activated. One of the most well-characterized and effective NK cell-activating receptors is the NK group 2D (NKG2D). NKG2D ligands, including major histocompatibility complex (MHC) class I polypeptide-related chain A (MICA) and B (MICB) sequence and the UL16 binding proteins (ULBPs) 1–6, are slightly expressed on normal tissues but are upregulated on tumor cells in response to cellular stress. DNAJ accessory molecule-1 (DNAM-1) and the natural cytotoxicity receptors (NCRs) Nkp30, Nkp44, and Nkp46 are other major NK cell-activating receptors. DNAM-1 ligands poliovirus receptor (PVR, CD155) and Nectin-2 (CD112) are overexpressed on many tumors, including sarcomas. The inhibitory receptors such as killer cell immunoglobulin-like receptors (KIRs) and the CD94–NKG2A regulate NK cell function by recognizing and binding to the classic and non-classic leukocyte antigen (HLA) class I molecules.

NK cells are potential effector cells against cancer. Higher numbers of tumor-infiltrating NK cells have been correlated with better outcome, and lower NK cell activity in the body has been associated with more risk of cancer development. However, NK cell therapy seems to be more effective for blood malignancies in which tumor cells are more accessible through the blood circulation than for solid tumors. Therefore, new approaches are needed to potentiate allogenic NK cell activity against solid tumors. Various agents such as immunomodulators, cytokines, and monoclonal antibodies can increase NK cell cytotoxicity against tumor cells. Tumor cells may escape NK cell recognition either by downregulating the ligands for NK cell activating receptors or suppressing NK cell activating receptor expression. Therefore, any strategies that can induce
expression of the ligands for NK cell activating receptors on tumor cells would lead to increased NK cell-mediated lysis.

Our team has had promising results in showing that NK cell therapy combined with aerosol interleukine-2 (IL-2) decreased OS lung metastasis tumor burden in a mouse model; this therapy, however, was not effective enough to completely cure OS lung metastasis. To optimize the efficacy of NK cell therapy in this study, we combined it with oral administration of entinostat (MS-275), a histone deacetylase (HDAC) inhibitor. We and other investigators have shown that the level of NK cell cytotoxicity is directly correlated with the density of its ligands for activating receptors expressed on tumor cells. Although HDAC inhibitors are mostly known by their immediate anticancer activity, accumulating evidence suggests that they also have immunomodulatory effects resulting in tumor cells being more recognized and targeted by immune cells. In this respect, for example, HDAC inhibitors increase tumor cell susceptibility to NK cell lysis by upregulating the ligands for activating receptors on tumor cells. We have shown previously that benzamidine containing HDAC inhibitor entinostat enhances NKG2D ligands on tumor cells that lead to more NK cell lysis. It also has been reported that HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA), sodium valproate, and entinostat upregulate NK cell ligand expression on tumor cells and make them more susceptible to NK cell cytotoxicity. Importantly, treatment with HDAC inhibitors does not increase the levels of NKG2D ligands in normal cells, suggesting a therapeutic advantage for the selective effect in tumors. Accordingly, we hypothesized that combining entinostat with NK cell therapy would increase the immunosensitivity of OS lung metastasis to NK cells.

In the present study, we investigated the effect of entinostat on immune sensitization of OS cells to NK cell-mediated lysis both in vitro and in our nude mouse human OS lung metastasis model. We demonstrated two potential mechanisms by which entinostat upregulates MICA and MICB gene expression in OS cells at the posttranscriptional level.

**Results**

**Entinostat increases OS cell susceptibility to NK cell-mediated cytotoxicity by upregulating ligands for activating NK cell receptors on OS cells**

To determine whether entinostat increase the sensitivity of OS cells to NK cell killing, we first investigated whether entinostat would increase the expression of ligands for NK cell receptors on OS cells. Four human OS cell lines (LM7, CCH-OS-D, CCH-OS-O, and KRIB) were treated with 2 μM entinostat (≤IC50) for 48 h and analyzed by flow cytometry. As shown in Fig. 1(A), entinostat treatment significantly upregulated ligands for NK cell-activating receptors but did not affect the ligand for the NK cell inhibitory KIR receptor (HLA-ABC). The upregulated ligands included CD155 (except for CCH-OS-D and KRIB), MIC A/B, ULBP1, and ULBP2/5/6. Since MICA and MICB are major ligands for activating receptor NKG2D, and the NKG2D–MICA/B interaction plays a major role in NK cell activation, we investigated the effect of entinostat on MICA/B mRNA and protein expression as well. Our results demonstrated that LM7 cells treated with entinostat showed increased mRNA (Fig. 1B) and protein expression (Fig. 1C) levels for MICA and MICB, in a dose dependent manner.

Next, we determined the stability of the increased ligands for NK cell receptors on OS cells in response to entinostat

Figure 1. Effect of entinostat on NK cell ligand expression on osteosarcoma (OS) cells and their susceptibility to NK cell-mediated cytotoxicity. (A) NK cell ligand expression on OS cells after incubation with 2 μM entinostat for 48 h. Data are shown as mean fluorescence intensity (MFI). (B) LM7 cells were incubated with 0, 0.5, 1.0, or 2 μM entinostat for 48 h, and total RNA was subjected to quantitative RT-PCR analysis using primers specific for MICA and MICB. (C) Protein levels of MICA/B from LM7 cell lysate were analyzed by Western blotting. (D) LM7 and CCH-OS-D cells were treated with media or entinostat (2 μM for 48 h). NK cell-mediated cytotoxicity was then quantified at various E/T ratio (0.3, 0.6, and 1.3) using a calcein release assay. (E) Cytotoxic activity of NK cells (control or pre-incubated with anti-NKG2D, NKp46, and DNAM blocking antibodies) against control LM7 cells or pre-treated with 2 μM entinostat for 48 h. p values < 0.05 are marked with *. All experiments were repeated three times, bars show mean ±/SEM.
Entinostat does not affect NK cell viability, receptor expression, or cytotoxic function

The ultimate goal of this study is to combine entinostat with NK cell therapy to augment the NK cell cytotoxic effect against OS lung metastasis in vivo; therefore, we next determined whether entinostat affected the viability of NK cells, NK cell receptor expression, and NK cell cytotoxic function. NK cells were expanded ex vivo for 4 weeks and treated with 0, 0.1, 0.5, 1.0, and 2.0 \( \mu M \) entinostat for 24 or 48 h. There was no effect on NK cell viability at either time point (Fig. 2A). With the exception of NKG2D, entinostat at \( \leq 2 \mu M \) for 24 h had no effect on NK cell receptor expression (Fig. 2B). However, at 48 h, downregulation of NKG2D, NKp30, NKp44, and NKp46 was induced by \( \geq 0.5 \mu M \) entinostat (Fig. 2B). DNAM-2 expression was not affected. These results suggest that for the in vivo study, administration of entinostat and NK cell treatment should be at least 24 h apart to avoid any adverse effects on NK cell receptor expression. The pre-treatment of NK cells with 2.0 \( \mu M \) entinostat for 24 h had no significant effect on NK cell-mediated cytotoxicity against LM7 and CCH-OS-D cells compared with control NK cells (Fig. 2C), confirming that entinostat does not abrogate NK cell functional activity within 24 h of treatment.

Entinostat regulates MICA/B expression by increasing acetylation of histone-4 linked to the MICA and MICB gene promoters

The molecular mechanism by which HDAC inhibitors immunosensitize tumor cells is not clearly known. Clarifying the mechanisms underlining the effect of HDAC inhibitors on tumor-immune recognition can lead to the development of their clinical use as anticancer drugs. Knowing that HDAC inhibitors increase activation of gene transcription by promoting histone acetylation on chromatin,\textsuperscript{23} we determined whether entinostat enhanced MICA and MICB gene transcription by acetylation of histone linked to the MICA and MICB gene promoters. Entinostat enhanced acetylated H3 (AcH3) and acetylated H4 (AcH4) expression in LM7 cells in a dose-dependent manner compared with untreated control cells (Fig. 3A). To determine whether increased transcription of MICA and MICB is mediated by enhanced acetylation of their gene promoters, lysates of control and LM7 cells treated with 2 \( \mu M \) entinostat for 48 h were analyzed by CHIP assays. Entinostat significantly increased acetylated H4 linked to the MICA and MICB promoters but had no significant effect on acetylation of H3 (Fig. 3B). 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.

Effect of entinostat on miR-20a, miR-93, and miR-106b expression

MicroRNAs (miRNAs) are classified as a large family of small, non-coding, and single-stranded RNAs that negatively regulate gene expression at the post-transcriptional level. miRNAs can inhibit gene expression by binding to the 3’ UTR of the targeted messenger RNA (mRNA) resulting in either inhibition of mRNA translation or its degradation.\textsuperscript{24} miR-20a, miR-93, miR-106b, miR-372, miR-373, and miR-520d have been shown to regulate MICA and MICB expression.\textsuperscript{25} We determined that OS cells do not express miR-372, miR-373, and miR-520d in OS cells (data not shown); therefore, the role of miR-20a, miR-93, and miR-106b in regulating MICA and MICB expression was examined. LM7 and KRIB cells were transiently transfected with the miR-20a, miR-93, and miR-106b mimics or control miRNAs. These cell lines were chosen for the study because of

| Ligands       | Untreated CCH-OS-D cells | 2 \( \mu M \) entinostat for 48 h | Time after drug removal |
|---------------|--------------------------|---------------------------------|-------------------------|
|               | 24 h                     | 48 h                            | 72 h                    |
| ULBP1         | 16.3                     | 29.6                            | 30.7                    |
| ULBP2/5/6     | 43.0                     | 85.8                            | 86.0                    |
| MICA/B        | 36.9                     | 61.5                            | 72.6                    |

| Ligands       | Untreated LM7 cells      | 2 \( \mu M \) entinostat for 48 h | Time after drug removal |
|---------------|--------------------------|---------------------------------|-------------------------|
|               | 24 h                     | 48 h                            | 72 h                    |
| ULBP1         | 7.9                      | 29                              | 24                      |
| ULBP2/5/6     | 77                       | 156                             | 150                     |
| MICA/B        | 151                      | 335                             | 306                     |

Table 1. Up-regulated NK cell ligands on OS cells treated with entinostat are stable for more than 24 h. LM7 and CCH-OS-D cells were treated with 2 \( \mu M \) entinostat for 48 h, and then the conditioned media were replaced with fresh media. Cells were harvested after 48 h of treatment and 24, 48, and 72 h after media was replaced. Cells were analyzed by flow cytometry with antibodies specific for MICA/B, ULBP1, and ULBP2/5/6. Data are shown as mean fluorescence intensity (MFI).
high levels of MICA and MICB expression on their surface (Fig. 1A). Overexpression of miRNA in transfected cell lines was confirmed by quantitative real-time polymerase chain reaction (RT-PCR) (Fig. S1). Cells were harvested at 24 and 48 h after transfection, and MICA and MICB expression levels were analyzed by flow cytometry. MICA and MICB expression was significantly downregulated by miR-20a, miR-93, and miR-106b in LM7 and KRB cells at 24 and 48 h after transfection (Fig. 4A and B). These results support the role of miRNAs (i.e., miR-20a, miR-93, and miR-106b) in regulating MICA and MICB expression in OS cells.

We next investigated whether entinostat regulates MICA and MICB expression by downregulating miR-20a, miR-93, and miR-106b. Three OS cell lines LM7, CCH-OS-D, and CCH-OS-O were treated with 2 μM entinostat for 48 h. Quantitative RT-PCR analysis revealed that all three miRNAs were significantly decreased in the entinostat-treated OS cells compared with the control samples (Fig. 4C). This was not a non-specific effect of entinostat, since entinostat had no effect on two other irrelevant miRNAs (miR-21 and miR-27a) that are expressed in OS cells (Fig. 4D).

We next determined whether entinostat upregulated MICA and MICB expression in OS lung metastasis in vivo by reducing miR-20a, miR-93, and miR-106b. A total of 2 × 10⁶ LM7 cells were injected intravenously into nude mice. After visible nodule formation in the lungs was documented, mice were treated with 5 mg/kg entinostat by oral/gavage three times a week for 2 weeks. The mice were then killed, total RNAs were extracted from lung nodules, and MICA and MICB mRNA levels as well as miRNA expression were evaluated by quantitative RT-PCR. Entinostat at 5 mg/kg, significantly increased MICA and MICB mRNA (Fig. 4E) and decreased miR-20a, miR-93, and miR-106b (Fig. 4F). These results suggest that entinostat treatment upregulates MICA and MICB expression by downregulating miR-20a, miR-93, and miR-106b expression in both OS cells and OS lung metastasis.

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

In this study, we first identified a subtherapeutic dose of entinostat that significantly increased the expression of NK cell ligands (MICA and MICB) on OS lung metastasis by injecting 2 × 10⁶ LM7 cells via the tail vein into nude mice. After visible nodule formation in the lungs was confirmed, the mice were treated with entinostat of 2.5, 5, or 10 mg/kg by oral/gavage 3 times a week for 2 weeks. We previously demonstrated that entinostat at 20 mg/kg inhibited the growth of OS lung metastasis. Therefore, the highest dose that 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. Both 5 and 10 mg/kg entinostat significantly enhanced the MICA and MICB mRNA levels; however, 2.5 mg/kg had no effect on either MICA or MICB expression (Fig. 5A). Thus, we chose to use 5 mg/kg entinostat in our combination therapy studies since this was the lowest dose that significantly increased MICA and MICB ligands.

For *in vivo* use, expanded NK cells were depleted for CD3+ T cells. NK cells receptor (NKG2D and CD16) expression was evaluated before injection and found to be ≥95% and ≥80% positive for NKG2D and CD16, respectively (data not shown). NK cells cytotoxicity against LM7 cells was also confirmed before infusion to ensure their killing function (data not shown).
We demonstrated above that entinostat sensitized OS cells to NK cell-mediated cytotoxicity by upregulating the ligands for NK cell activating receptors \textit{in vitro}. Therefore, we anticipated that combining entinostat with NK cell therapy would increase the therapeutic efficacy of NK cells against OS lung metastasis. LM7 cells were injected and pulmonary micrometastasis formation was confirmed at week 5. Treatment was then initiated with DMSO, entinostat, NK cells, or NK cells + entinostat for 5 weeks (n = 12/16/group). Entinostat was administered twice a week, and NK cells were infused 3 times a week. As demonstrated above, upregulated ligands on OS cells (MICA/B, ULBP1, and ULBP2/5/6) by entinostat are stable for more than 24 h (Table 1), indicating that the 2 treatments can be separated by 24 h without loss of NK cell ligand expression on the tumor cells. Furthermore, we showed that 48 h but not 24 h of treatment with entinostat downregulated NK cell receptor expression including NKG2D, NKp30, NKp44, and NKp46 (Fig. 2B). 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). Fifty million NK cells injected each time were well tolerated, and no infusion-related or long-term adverse effects were detected after 5 weeks of treatment. Combining entinostat with NK cell therapy failed to enhance the therapeutic effect. The combination therapy did not reduce either the number or the size of OS lung nodules (Fig. 5Band C).

Entinostat increased MICA/B expression on OS lung metastasis

To determine whether entinostat upregulated MICA/B expression on OS lung metastasis, tumor sections were analyzed by immunohistochemical staining for MICA/B. The expression of MICA/B was significantly upregulated in the tumor nodules from mice treated with entinostat alone or entinostat + NK cells (Fig. 6). These data indicate that the therapeutic failure was not secondary to the inability of entinostat to upregulate MICA/B.

Infused NK cells migrated to the lung but did not penetrate into the lung tumor nodules

The efficacy of NK cell therapy depends on the cells’ ability to migrate to the organ, where metastasis are present, and penetrate into the tumor. To confirm that the infused NK cells migrate to the lungs, nude mice were injected with CM-DiL–labeled NK cells. At 50 min and at 24 h after NK cell injection, the mice were killed, and lung sections were examined under a fluorescent microscope. High numbers of NK cells were visualized in the lung 50 min after injection; however, by 24 h few NK cells were left in the lung (Fig. 7A). This data confirmed that NK cells migrated to the mouse lungs but either exited or died within 24 h. We further investigated whether after migration, NK cells penetrated into the OS lung metastasis. Tumor
sections from our in vivo experiment shown in Fig. 5(B and C) were analyzed by immunofluorescence staining for NK cells.

As shown in Fig. 7(B), NK cells were only observed at the periphery of the nodules, but not inside. 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.

**Discussion**

Expression of the ligands for NK cell-activating receptors on tumor cells play a critical role for tumor cells being recognized and killed by NK cells; therefore, using an agent that is non-toxic to NK cells and upregulates the ligands on tumor cells...
can be considered a potential approach to enhance NK cell function. We demonstrated that the HDAC inhibitor entinostat increased ligands for NK cell activation receptors but not ligands for inhibitory receptors on OS cells in vitro, and this led to more NK cell-mediated cytotoxicity against OS cells. This observation is in agreement with other studies that have shown HDAC inhibitors, including entinostat sensitize tumor cells to NK cell cytotoxicity with the same mechanism.\textsuperscript{20-22,27}

We also demonstrated that the upregulated ligands are stable for more than 24 h after entinostat is removed from the culture, suggesting that the effect of the drug on ligand expression \textit{in vivo} would be unchanged for a while, even while the drug is being metabolized and after it is removed from the body. Considering that the 48-h treatment with entinostat downregulated NK cell-activating receptors expression \textit{in vitro} (Fig. 2B), this window of time is important because it provided the possibility to administer entinostat and NK cells in a different time point \textit{in vivo} to avoid any adverse effects on NK cell receptor expression.

Importantly, we showed that 24-h treatment with 2 \( \mu \) M entinostat did not change the NK cell cytotoxicity against OS cells. However, other studies showed that sodium valproate (a broad-spectrum class I- and IIa-specific HDAC inhibitor), SAHA (a pan-HDAC inhibitor), and romidepsin (a class 1 selective HDAC inhibitor) have inhibitory effects on NK cell cytotoxicity.\textsuperscript{28,29} 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

### Table 2

Experimental plan for combination therapy with entinostat and NK cells in mice with OS lung metastasis. Mice were treated with entinostat (5 mg/kg) by oral/gavage three times a week for 5 weeks. \( 5 \times 10^6 \) NK cells were injected twice a week for 5 weeks. NK cell therapy was 24 h following entinostat administration.

| Groups            | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday |
|-------------------|--------|---------|-----------|----------|--------|----------|--------|
| Control           | DMSO   |         |           | DMSO     |        |          |        |
| Entinostat        | Entinostat |       |           | Entinostat |        |          |        |
| NK cells          | DMSO   | NK cells |           | DMSO     | NK cells |          | Entinostat |
| NK cells + entinostat | Entinostat | NK cells |           | Entinostat | NK cells |          | Entinostat |
their ability to inhibit the function of a specific HDAC. Moreover, in the previous studies, fresh NK cells had been used in the experiment whereas we used 4-week-old expanded NK cells, which showed more activated phenotypes and cytotoxicity than fresh NK cells do. Presently though, the reason for the disparity among these studies is unclear.

We further investigated the mechanism by which entinostat regulates MICA/B expression in OS cells from the epigenetics and miRNA regulation point of view. Acetylation of both H3 and H4 is associated with unfolding the chromatin structure and enhanced gene transcription. Here, we showed for the first time that MICA and MICB gene expression increased in OS cells because of the direct effect of entinostat on promoting acetylation of H4 (but not H3) linked to MICA and MICB gene promoters. This may be unique to OS cells since previous reports showed that treating colorectal adenocarcinoma cells with entinostat resulted in upregulation of MICA and MICB expression by acetylation of H3 linked to MICA and MICB gene promoters. Entinostat could also indirectly upregulate MICA and MICB by downregulating miRNAs targeting MICA/B (miR-20a, miR-93, and miR-106b). Similar results have been reported in hepatocellular carcinoma by HDAC inhibitor SAHA. More studies are required to understand the regulatory mechanism of entinostat in suppressing miRNA expression. In conclusion, our results suggest that entinostat not only targets histone proteins to control MICA/B gene transcription, but also may target non-histone protein, and both mechanisms contribute to MICA/B increased expression.

Although our finding demonstrated that entinostat increased NK cell ligands (MICA and MICB) in OS lung metastasis, its combination with NK cell therapy neither augmented NK cell-mediated cytotoxicity against OS pulmonary metastasis nor improved the survival rate. The infused NK cells failed to infiltrate into the lung nodules, in spite of penetration into the lungs. This may explain why entinostat along with NK cells had no additive effect on tumor regression. There might be various reasons for the failure of NK cells to penetrate the lung nodules or lead to their apoptosis after penetration. It is known that, ex vivo activated NK cells have a limited survival time and show low tumor penetration without cytokine support in vivo. We used nude mice in which the crosstalk between NK cells and T cells is absent due to the lack of T cells in nude mice. The critical role of CD4+ T cells in helping NK cell function has been well identified. CD4+ T cells are known to be the main source for IL-2 production, and IL-2 plays an important role in NK cell proliferation, activation, and survival. Recent studies have demonstrated the critical role of CD4+ T and NK cell interaction in immune cell cancer defense. Adptive transfer of IL-12/15/18–preactivated NK cells in lymphoma or melanoma-bearing mice did lead to tumor regression in the presence of CD4+ T cells that produce IL-2. IFNγ-mediated tumor rejection caused by NK cells is dependent on the presence of CD4+ T cells and their IL-2. We have also previously shown that adding aerosol IL-2 to NK cell therapy dramatically increased the efficacy of NK cells against OS lung metastasis in nude mice and significantly improved the overall survival rate. Besides increasing the number of infiltrated NK cells in the lung nodules, aerosol IL-2 resulted in NK cell proliferation in the lungs. Based on another study, NK cell immunotherapy in NSG mice-bearing OS resulted in lower burden in the tibia, reduced bone damage, and raised overall survival when combined with IL-2 IP injection. These findings suggest that NK cells are highly dependent on cytokine milieu support; therefore, adding aerosol IL-2 or IL-21, another stimulator of NK cell-mediated cytotoxicity, to our treatment may be beneficial for persistence in the lung, penetration into the tumor, and enhanced cytotoxic function of infused NK cells in vivo.

However, other inhibitory factors may have also been involved. Proteolytic shedding of NKG2D ligands such as MICA and MICB from tumor cells has been described as an

Figure 6. Entinostat increased MICA/B expression on OS lung metastasis. (A) Paraffin-embedded lung tissues were analyzed for MICA/B expression with use of immunohistochemical staining. (B) Mean MICA/B positivity was calculated in five random fields per section using Simple PCI software.
escape mechanism from cell immunity defense. High levels of soluble MICA (sMICA) in OS patients' serum has indeed been detected and has been correlated with disease stage and metastasis rate. However, more studies are needed to reveal whether sMICA/sMICB had interfered with our NK cell therapy. The complex immunosuppressive character of solid tumors has a crucial inhibitory effect on immune system defense against tumor progression. For instance, programmed death-ligand 1 (PD-L1) upregulation on tumor cells, including OS has been reported. PD-L1/PD-1 interaction can inhibit the antitumor activity of immune cells by triggering inhibitory signals into the immune cells. This inhibitory effect could be more relevant in our study because the in vitro results showed that entinostat upregulated PD-L1 expression on OS cells in a dose-dependent manner (data not shown). However, the levels of PD-L1 on OS lung metastasis and of PD-1 on infused NK cells expression have not been determined in our study. Other immunosuppressive factors/cells such as TGF-β, IL-10, macrophage migration inhibitory factor (MIF), and myeloid-derived suppressor cells (MDSCs) might have suppressed NK cell activation in the OS lung metastasis microenvironment. It has been demonstrated that TGF-β1 secreted by tumor cells can decrease NKG2D and Nkp30 expression on NK cells. Overexpression of MIF on ovarian cancers is associated with the downregulation of NKG2D on NK cells.
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 since various parameters are involved. This emphasizes the fact that NK cell therapy for solid tumor is still a challenging field. To improve NK cell therapy against solid tumors, we need to optimize NK cells infiltration into and maintenance in the tumor nodules as well as to better sustain NK cell activation in the tumor microenvironment. These challenges regarding cellular immunotherapy against solid tumors may be overcome by combination therapy.

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-histone H3 antibody, and anti-human acetyl-histone H4 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, 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 seven times through the lungs of nude mice. KRIIB, 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 non-essential 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.

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 animal protocols approved by the Institutional Animal Care and Use Committee (IACUC).

Human NK cell isolation and expansion

Human NK cells were purified from healthy donors’ buffy coat after informed consent (Gulf Coast Regional Blood Center, Houston, TX). Isolated NK cells were expanded in vitro for 4 weeks with use of recombinant human IL-2 and genetically engineered K562, as described previously. By end of 4 weeks of expansion, NK cell purity was ≥99%. Expanded NK cells were frozen and kept at −80°C for further use. After being thawed and before being infused into mice, NK cells were kept in culture and treated with IL-2 for at least 48 h. Thereafter, their viability and receptor expression were evaluated with use of Vi-CELL and flow cytometry, respectively. Furthermore, to confirm that 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 FACS Calibur 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. Cells were harvested and lysed with RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Santa Cruz Biotechnology, Inc.). Total protein concentration was measured by using a bicinchoninic acid assay kit (Bio-Rad Laboratories); bovine serum albumin was used as a standard. Western blot analysis was performed as described previously. A mouse monoclonal anti-human antibody MICA/B was used to detect MICA/B protein expression. Rabbit anti acetyl-histone3, or acetyl-histone4 were used to detect acetylated H3 or acetylated H4.

Quantitative RT-PCR analysis

LM7 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 Reverse Transcription System with oligo-dT primer (Promega Corporation, Madison, WI) according to the manufacturer’s instructions. mRNA levels of MICA and MICB were measured by RT-PCR by using iQ SYBR Green Supermix (Bio-Rad Laboratories), bovine serum albumin was used as a standard. mRNA levels of MICA and MICB were measured by RT-PCR by using iQ SYBR Green Supermix (Bio-Rad Laboratories). Specific primers for MICA and MICB were as follows: MICA, 5’- AGGTTTTCCTTGAGGTACA-3’ (forward) and 5’-GGTCTCTCGTCCCAGTTA-3’ (reverse); MICB, 5’-TCTTCGGTACAACCTCATGTG-3’ (forward), and 5’-TCCCCAGGTTCTAGTCCAG-3’ (reverse). Cycling conditions were 95°C for 3 min, 45 cycles of 59°C annealing for 30 sec, 95°C for 30 sec, 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. The resulting cDNA was subjected to PCR amplification with use of specific primers for miR-20a, miR-93, or miR-106b.

Cytotoxicity assay

NK cell cytotoxicity was measured by using a calcein release assay as described previously. Briefly, $1 \times 10^6$ target cells were resuspended in $1 \mu M$ calcein-AM diluted in DMED and incubated at $37^\circ C$ 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 $200 \mu L$ in triplicate and at a ratio of $0.3125:1-10:1$ (effector cell: target cell). Plates then were incubated at $37^\circ C$ 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 $100 \mu L$ of supernatant was transferred to a 96-well flat bottom plate. The plate was read at excitation and emission wavelengths of $485 \text{ nm}$ and $530 \text{ nm}$, respectively. Percent-specific lysis was calculated according to the formula $[(\text{test release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})] \times 100$.

Chromatin immunoprecipitation (CHIP)

LM7 cells were treated with $2 \mu M$ 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 twice and co-cultured with ex vivo expanded NK cells in 96-well U-bottom plates in a total volume of $200 \mu L$ in triplicate and at a ratio of $0.3125:1-10:1$ (effector cell: target cell). Plates then were incubated at $37^\circ C$ 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 $100 \mu L$ of supernatant was transferred to a 96-well flat bottom plate. The plate was read at excitation and emission wavelengths of $485 \text{ nm}$ and $530 \text{ nm}$, respectively. Percent-specific lysis was calculated according to the formula $[(\text{test release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})] \times 100$.

miRNA mimics transfection

miRNA mimics and the negative control of miRNA mimics were transfected into OS cells lines by 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 $5 \text{ pmol}$. Cells were collected at 24 and $48 \text{ h}$ 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 RT-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 $0.2 \text{ mL}$ of PBS solution. Visible nodule formation was confirmed 8 weeks later after euthanization. Mice then were treated with 2.5, 5, or $10 \text{ mg/kg}$ entinostat three times a week for 2 weeks. At the end of treatment, nodules were removed from the lungs, and total miRNAs were extracted with use of Trizol reagent. MICA and MICB mRNA levels were evaluated by quantitative RT-PCR.

Animal model

Four- to six-week-old nu/nu mice were injected with $2 \times 10^6$ LM7 cells. About 5–6 weeks later, three mice were killed to evaluate lung micrometastasis formation. Thereafter, treatment was started in the following four mouse treatment groups: (1) DMSO; (2) entinostat; (3) NK cells; and (4) NK cells + entinostat. Mice received $5 \text{ mg/kg}$ of entinostat or $200 \mu L$ of DMSO (control) by oral gavage three times a week for 5 weeks. A total of $50 \times 10^6$ 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 killed after 5 weeks of treatment. The lungs were removed, and the visible lung metastases were counted and measured. Fresh-frozen and paraffin-embedded samples from the resected lungs were prepared for further use.

In the case of using luciferase-expressing LM7 cells, nude mice were injected with $2 \times 10^6$ luciferase-expressing LM7 (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 performed similar to the first in vivo study for 5 weeks. A total of 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 $3\%$ $H_2O_2$ for 12 min.
followed by PBS supplemented with 4% fish gelatin in PBS. Antibodies specific to MICA/B (Abcam, MA) was applied and left overnight at 4 °C followed by incubation with horse radish peroxidase-labeled secondary antibodies for 2 h 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 not treated with primary antibodies.

**Immunofluorescence staining**

Frozen sections from our *in vivo* experiment were analyzed by immunofluorescence staining for NK cells. Slides were stained with Goat anti-human NKP46 antibody (10 μg/mL) from R&D System, MN. The nuclei were also counterstained with use of Hoechst 33,342 nucleic acid stain. Slides then were mounted and examined under a fluorescence microscope.

To quantify the presence of infused NK cell in the mouse lung, 50 × 10⁶ 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 killed 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.

**Statistical analysis**

The unpaired student *t* test 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.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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