Original Article
Epigenetic suppression of the antitumor cytotoxicity of NK cells by histone deacetylase inhibitor valproic acid

Xiumin Shi¹, Min Li¹, Meizi Cui¹, Chao Niu¹, Jianting Xu¹, Lei Zhou¹, Wei Li¹, Yushun Gao³, Weisheng Kong⁴, Jiwei Cui¹, Jifan Hu¹,², Haofan Jin¹

¹Stem Cell and Cancer Center, First Hospital, Jilin University, Changchun, Jilin 130021, China; ²Stanford University Medical School, Palo Alto Veterans Institute for Research, Palo Alto, CA 94304, USA; ³Department of Thoracic Surgical Oncology, Cancer Institute, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100021, China; ⁴BASO Cell Science & Technology Co., Ltd, Zhuhai, Guangdong 519015, China

Received November 30, 2015; Accepted December 24, 2015; Epub February 15, 2016; Published March 1, 2016

Abstract: Natural killer (NK) cells play an essential role in the fight against tumor development. The therapeutic use of autologous NK cells has been exploited to treat human malignancies, yet only limited antitumor activity is observed in cancer patients. In this study, we sought to augment the antitumor activity of NK cells using epigenetic approaches. Four small molecules that have been known to promote epigenetic reprogramming were tested for their ability to enhance the activity of NK cells. Using a tumor cell lysis assay, we found that the DNA demethylating agent 5-azacytidine and vitamin C did not significantly affect the tumor killing ability of NK cells. The thyroid hormone triiodothyronine (T3) slightly increased the activity of NK cells. The histone deacetylase inhibitor valproic acid (VPA), however, inhibited NK cell lytic activity against leukemic cells in a dose-dependent manner. Pretreatment using VPA reduced IFNγ secretion, impaired CD107a degranulation, and induced apoptosis by activating the PD-1/PD-L1 pathway. VPA downregulated the expression of the activating receptor NKG2D (natural-killer group 2, member D) by inducing histone K9 hypermethylation and DNA methylation in the gene promoter. Histone deacetylase inhibitors have been developed as anticancer agents for use as monotherapies or in combination with other anticancer therapies. Our data suggest that the activity of histone deacetylase inhibitors on NK cell activity should be considered in drug development.

Keywords: NK cell, valproic acid, expression, DNA methylation, histone methylation, epigenetics, tumor killing, apoptosis

Introduction
There is growing interest in the potential of exploring adoptive cellular immunotherapy to treat tumors by restoring immunity in cancer patients. Natural killer (NK) cells, defined as CD3-CD56⁺ lymphocytes, are a critical component of the innate immune system and play an essential role in the fight against tumor development and viral infection [1, 2]. Although representing only approximately 10-15% of all lymphocytes in the peripheral blood, NK cells can directly lyse tumor cells without prior sensitization as is commonly required for T lymphocytes [2, 3]. Cytokines secreted by NK cells provide adaptive immune response through a mechanism of intrinsic immune-surveillance [4, 5]. Mechanistically, NK cells target tumor cells through a delicate balance between the activating (killer activating receptor, KAR) and the inhibitory (killer immunoglobulin-like receptors, KIR) signal pathways mediated through membrane receptors [2, 6]. Once activated, NK cells exert their tumor-lytic activity to control tumor growth.

NK cells derived from the peripheral blood of cancer patients have been explored as potential agents for adoptive cell therapy in cancer. Unfortunately, tumor infiltrating NK cells freshly isolated from patients are not cytotoxic to autologous tumors [7]. Similarly, NK cells from the peripheral blood of cancer patients have significantly reduced cytotoxic activity, probably due to interference through the membrane KIR receptors that interact with autologous major histocompatibility complex (MHC) antigens [3, 8]. Consequently, infusions of patients’ autolo-
gous NK cells have not shown significant clinical benefit to eradicate tumors [7]. Several drugs can augment the function of NK cells, including cytokines and antibody drugs that target PD1, KIR, and tumor antigens [2]. After activation and expansion with cytokines, NK cells may readily lyse tumor cells that express self-MHC molecules. As demonstrated recently, T cells can be engineered to express a chimeric antigen receptor (CAR) that recognizes a specific antigen molecule on tumor cells [9]. This approach has demonstrated potent clinical efficacy as it could induce remissions in patients with advanced leukemia [10, 11]. However, application of the CART-T cell therapy to other cancers has been challenging [12], primarily due to life-threatening cytokine-release syndromes, antigen-nonspecific toxicities to normal tissues, and low transduction efficiency of primary T cells by viral vectors.

Accumulating evidence has demonstrated the presence of epigenetic mechanisms that control tumor immunity. For example, tumor cells escape immune destruction by epigenetically suppressing immune genes, including MHC class II, CD40, MHC class I, components of the class I peptide presentation pathway (TAP1, TAP2, LMP2, LMP7, Erp57 and Tapasin), B7-1/2, NKG2D ligands and certain tumor antigens [13-15]. Similarly, epigenetic approaches involving DNA methylation and histone modifications have been explored as a means to regulate the expression of key immune system-related genes, thus modifying the development of the immune responses [16, 17]. However, it is unclear whether these epigenetic approaches can be employed to boost the antitumor therapy mediated by NK cells.

In this study we examined whether several small molecules that have been known to promote reprogramming of somatic cells into pluripotent stem cells [18-21] could epigenetically activate NK cells. We are particularly interested in the role of those epigenetic modifying chemicals, such as inhibitors of HDAC (histone deacetylase) and DNMT (DNA methyltransferase), which have already been approved by FDA for clinical treatment of myelodysplastic syndromes and acute myeloid leukemia.

Materials and methods

Cell culture

Human erythroleukemic K562, acute T cell leukemia Jurkat, and hepatoma HepG2 cells were purchased from the American Type Culture Collection (ATCC, VA) and routinely cultivated in RPMI-1640 medium plus 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml streptomycin-penicillin in a humidified atmosphere containing 5% CO₂.

Isolation and expansion of NK cells

The study protocol was approved by the Research Ethics Board of the First Hospital of Jilin University. This study was carried out at the First Hospital of Jilin University (Changchun, Jilin, China) according to Declaration of Helsinki principles. Informed consent was obtained from cancer patients [22, 23]. Patients with non-small cell lung cancer were diagnosed based on the AJCC TNM staging system (7th edition, 2009) [24] and the disease stage of small cell lung cancer was grouped according to the system by the Veterans’ Administration Lung Study Group [25] (Table 1).

Peripheral blood mononuclear cells (PBMCs) were isolated from cancer patients by Ficoll (Lymphoprep) density gradient centrifugation [22] and were cultured in AlyS 505 NK-EX (CSTI, Japan), supplemented with 300 U/ml IL-2 and 10 ng/ml anti-CD3 Monoclonal antibodies. Flow cytometric analysis showed the purity of NK cells (CD56⁺CD3⁻) was >95% of the isolated cells. NK cells were cultured at a density of 2×10⁶ cells/ml in six-well plates in a humidified atmosphere with 5% CO₂ at 37°C.

For epigenetic treatment, NK cells were treated with valproic acid (VPA, Sigma, MO), vitamin C (Vit-C, Sigma, MO), 5-azacytidine (5-AzaC, Sigma, MO), and T3 (Santa Cruz Biotechnology, CA) at the indicated concentrations for 24 hr. NK cells were treated with PBS as the negative control.

NK cytotoxicity assay

NK cell cytotoxicity was determined using the calcein release assay (Fisher Scientific, CA), a fluorometric assay comparable to the chromium release assay in determining NK cell cytotoxicity [26]. After treatment with VPA (0.5 mM, 1 mM, 2 mM, 4 mM), T3 (20 µM), Vit-C (1 mM), and 5-AzaC (2 µM) for 24 h, NK cells (effector cells) were tested for cytotoxicity by incubating with target cells (K562, Jurkat, HepG2) that were labeled with 1 µg/ml calcein-AM (Dojindo Laboratories, Japan) for 30 min at 37°C with
VPA epigenetically suppresses NK cell cytotoxicity

occasional shaking. Effector cells and target cells were co-cultured at the indicated effector:target (E:T) ratios (ranging from 5:1 to 40:1) and incubated at 37°C for 4 h. After incubation, 100 μl of the supernatant was harvested and transferred to a new plate. Absorbance at 485 nm of excitation light wavelength and 528 nm of emission wavelength was determined using a Synergy™ HT multi-function MPP detector (BioTek, VT). The percent lysis was calculated according to the formula \[
\left(\frac{\text{experimental release}-\text{spontaneous release}}{\text{maximum release}-\text{spontaneous release}}\right)\times 100.
\]

**Flow cytometry analysis**

Surface expression of NKG2D and CD107a was analyzed with a FACS Calibur flow cytometer (BD Biosciences) using following mAbs: APC-anti-NKG2D, PE-anti-CD56, PerCP-anti-CD3, APC-anti-CD107a and isotype-matched mAb (negative control for non-specific binding) (all from BD Biosciences, CA). The proportions of living, dead and apoptotic cells were determined with PI and Annexin-V-FITC apoptosis detection kit (Beyotime).

**Measurement of INF-γ secretion**

NK cells were previously treated with VPA at indicated concentrations (0.5 mM, 1 mM, 2 mM, 4 mM) for 24 h at the density of \(2\times10^6\) cells/ml. The supernatant was harvested and IFN-γ production was assayed using the Human IFN-γ ELISA Kit (Boster, Wuhan, China), according to the manufacturer’s instructions.

**Quantitative real-time reverse transcription PCR (qRT-PCR)**

Total RNA was extracted from human cultured primary NK cells by TRI-REAGENT (Sigma, MO), according to the manufacturer’s guide. After removing genomic DNA contamination with DNase I (Sigma, MO), M-MLV Reverse Transcriptase (Invitrogen, CA) was used to synthesize cDNA. Quantitative real-time PCR was performed in triplicate with CFX96TM real-time system (BIO-RAD) using SYBR Prime Script TM RT-PCR Kit (Takara, CA). DNA was amplified using specific primers (Table S1). The mRNA expression level of NKG2D were calculated using threshold cycle (Ct) values standardized to β-ACTIN (housekeeping gene), applying the \(2^{-\Delta\Delta C_{t}}\) method [23, 27].

**Western blot analysis**

Western blotting was used to measure proteins and phosphoproteins as previously described [28, 29]. Briefly, NK cells were washed with ice-cold PBS and lysed at 4°C on ice for 20 min in 100 μl lysis buffer (50 mM, pH 7.5 Tris-HCl,
VPA epigenetically suppresses NK cell cytotoxicity

DNA methylation by bisulfite sequencing

Genomic DNA was isolated using the DNeasy® Blood and Tissue kit (Qiagen) and modified with bisulfate using the EZ-DNA Methylation Gold™ kit (Zymo Research), according to the manufacturer’s instructions. DNA was amplified using specific primers (Table S1). PCR products were cloned by pJET PCR Cloning kit (K1231, Thermo Scientific, CA) and ten independent clones from each sample were sequenced to determine the status of DNA methylation [30].

ChIP assays

ChIP assays were performed using the Pierce™ Agarose Chip Kit (Thermo Scientific, CA), according to the manufacturer’s instructions. Specific anti-trimethyl-H3K4 (9727) and anti-dimethyl-H3-K9 (9753) antibodies were used to determine histone methylation and normal rabbit IgG was used as a negative control. All antibodies were obtained from Cell Signaling Technology. DNA was extracted and analyzed by qRT-PCR with specific primers (Table S1). Enrichment was calculated as the percentage of input DNA using the formula: % INPUT = 2 \exp \left[ \frac{Ct (BOUND) - (Ct(UNBOUND) - \log_2 \text{(UNBOUND DILUTION FACTOR))} \times 100. \right]

Statistical analysis

All statistical tests were performed using SPSS version 15.0 (SPSS Inc.). Comparisons between groups were performed with the non-parametric Wilcoxon rank test or Student’s t-test when data were normally distributed. The comparative CT method was applied in the quantitative real-time RT-PCR assay according to the delta-delta CT method [31, 32]. Data were considered statistically significant at P≤0.05.

Results

Modulating effect of reprogramming chemicals on tumor lysis activity of NK cells

The escape from immune surveillance by cancer cells is controlled by epigenetic mechanisms that collaborate with genetic mutations in determining tumor progression [33].
Lymphocytes from cancer patients can be reprogrammed to control the development of tumors [34, 35]. We proposed to augment tumor cytotoxicity of NK cells by using small molecules that have been shown to improve cell reprogramming through epigenetic mechanisms, including 5-AzaC (DNA demethylation [18]), Vit-C (a micronutrient that modifies the activity of histone demethylating dioxygenases in reprogramming [36]), T3 (a thyroid hormone that promotes reprogramming by activating the PI3K/AKT signal pathway [21]), and VPA (a histone deacetylase inhibitor [20]). NK cells were isolated by flow cytometry selecting for the CD56+CD3- surface marker and were treated with reprogramming chemicals for 24 hr, using concentrations that have been proved to promote reprogramming (T3 20 µM, Vit-C 1 mM, 5-AzaC 2 µM, VPA 1 mM, respectively). After treatment, NK cells were tested for their activity to lyse tumor cells at various effector:target (E:T) ratios. Human K562 erythroleukemia cells were used as the target cell. As shown in Figure 1A, the thyroid hormone T3 slightly increased the specific lysis of K562, but the difference was not statistically significant. Vit-C and 5-AzaC did not affect the ability of NK cells to lyse tumor cells. However, the histone deacetylase inhibitor VPA significantly reduced NK cell cytotoxicity at all effector:target (E:T) ratios tested (p < 0.05). We also examined the role of these reprogramming compounds in Jurkat (acute T cell leukemia, Figure 1B) and HepG2 (hepatoma, Figure 1C) tumor cell lines. Again, the histone deacetylase inhibitor suppressed the antitumor cytotoxicity of NK Cells in both tumor cells. In addition, we also test whether the effect of these small compounds on cytotoxicity of NK cells were affected by cytokines used in the medium. For this, we treated NK cells with small compounds in the absence of cytokines and anti-CD3 monoclonal antibody. We found that removal of the cytokine (IL2) in the medium during the exposure to small compounds did not significantly impact the role of small compounds in NK cells (Figure S1).

**VPA dose-dependently inhibits the oncolytic activity of NK cells**

To further define the role of VPA, we treated NK cells with varying concentrations of the histone deacetylase inhibitor and assessed tumor lysis activity in K562 cells. We found that the cytotoxicity of VPA-treated NK cells against K562 cells was reduced in a concentration-dependent manner (Figure 2A).

We also examined if NK cells would resume their tumor-killing activity after the withdrawal of VPA (Figure 2B).

**VPA induced apoptosis of NK cells**

To delineate the mechanisms underlying the attenuated cytotoxicity of NK cells by VPA, we first used FACS to measure apoptosis in the
VPA epigenetically suppresses NK cell cytotoxicity

VPA 0 mM

![VPA 0 mM](image)

VPA 0.5 mM

![VPA 0.5 mM](image)

VPA 1.0 mM

![VPA 1.0 mM](image)

VPA 2.0 mM

![VPA 2.0 mM](image)

VPA 4.0 mM

![VPA 4.0 mM](image)

positive control

![positive control](image)

Figure 3. VPA induced apoptosis in NK cells. NK cells were treated with 0.5 mM, 1 mM, 2 mM, and 4 mM of VPA. After PI/Annexin-V staining, cell viability and apoptosis were measured by FACS.

treated cells. NK cells were treated with 0.5 mM, 1 mM, 2 mM, and 4 mM of VPA. Cell viability was measured by PI/Annexin-V staining. We found that the percentage of PI+/Annexin-V+ cell increased slightly (7.35%, 8.38%, 10.6%, 13.1%) as compared with the PBS control (5.15%) (Figure 3). These data suggest that the induction of apoptosis may be involved in a small portion, if not all, of the attenuated cytotoxicity in NK cells.

VPA downregulates CD107a and impairs IFN-γ secretion

CD107a, also called lysosomal-associated membrane protein-1 (LAMP-1), is a reliable biomarker of CD8+ T-cell degranulation following stimulation [37]. CD107a is also a sensitive marker of NK cell activity, as it is significantly upregulated on the surface of NK cells following stimulation with MHC devoid targets. The expression of CD107a correlates with NK cell-mediated lysis of target cells [38].

We thus quantified the degranulation pathway by measuring CD107a in VPA-treated cells. CD107a expression was analyzed by flow cytometry after incubation of NK cells using APC-anti-CD107a. We found impaired degranulation as demonstrated by the reduced CD107a expression after treatment with VPA (Figure 4A). These results indicate that VPA treatment not only induces NK cells apoptosis but also impairs the production of other molecules essential for the lytic activity of cytotoxic NK cells.

After recognition of target cells, NK cells release cytolytic granules containing granzyme B (GZMB) and perforin (PRF1), and produce IFN-γ, contributing to the cytolysis of target cells. As seen in Figure 4B, IFN-γ secretion decreased in NK cells treated with VPA in a dose-response manner, suggesting that histone acetylation significantly impaired cytokine production in association with attenuated cytotoxicity of NK cells. Similarly, treatment of NK cells with VPA also downregulated the activity of the STAT5 interferon pathway (Figure 4C).

Downregulation of PD-1 and PD-L1 by VPA in NK cells

Programmed death-1 (PD-1) and its ligand PD-L1 are inhibitory signaling molecules
VPA epigenetically suppresses NK cell cytotoxicity

involved in the immune escape mechanisms in cancer [39, 40]. We quantitated the expression of PD-1 and PD-L1 in VPA-treated NK cells. In PBS-treated NK cell controls, both PD-1 and PD-L1 were constitutively expressed at very low levels. After treatment with 2 mM VPA, however, we found that both PD-1 and its ligand PD-L1 were significantly upregulated (Figure 5), suggesting a role of the PD-1 inhibitory pathway in VPA-mediated suppression of NK cell cytotoxicity.

**VPA downregulates NKG2D in NK cells**

The cytotoxicity of NK cells is dependent on activating NK receptor systems NKG2D (natu-
VPA epigenetically suppresses NK cell cytotoxicity

To examine whether the expression of NKG2D is influenced at the transcriptional level upon VPA treatment, we examined gene expression patterns of NKG2D in NK cells. Real-time RT-PCR results revealed that the decline of NKG2D mRNA expression was VPA concentration-dependent (Figure 6C). We also observed a similar decline of NKG2D proteins using Western blot following the treatment of VPA (Figure 6D). Collectively, these data suggest that VPA-mediated downregulation of NKG2D may be an important mechanism underlying the suppression of lytic capacity of NK cells.

Epigenetic regulation of NKG2D by VPA

The decline of NKG2D expression was primarily due to reduced transcription of the NKG2D gene. In view of this, we determined whether epigenetic mechanisms, such as DNA methylation, could be involved in regulating the NKG2D gene. The NKG2D promoter contains a total of 9 CpG sites around the translation initiation site (between -2561 and -187 positions) (Figure 7A). We analyzed the status of DNA methylation in this CpG island by bisulfite sequencing and correlated DNA methylation with the gene activity of NKG2D in NK cells isolated from three cancer patients. As seen in Figure 7B, there was a slight increase in DNA methylation in the NKG2D promoter in VPA-treated NK cells. These results suggest that DNA methylation contributes to only a small fraction of the downregulation of the NKG2D gene.

We then examined whether histone methylation (H3K9me2 and H3K4me3) was involved in regulating NKG2D expression. After treatment with 2 mM VPA for 24 h, we collected cells and used ChIP assay to determine the level of H3K9me2 and H3K4me3 in the NKG2D gene. We observed a significant increase in H3K9me2 levels at the NKG2D locus in VPA-treated NK cells as compared with untreated NK cells (Figure 7C). H3K4me3 levels decreased slightly, but the difference was not statistically significant. Taken together, these results suggest that the increase of H3K9me2 in the NKG2D gene promoter may correlate with the decreased expression of NKG2D receptor on the cell surface of NK cells.

Discussion

Data from clinical trials demonstrate that infusions of autologous NK cells are very well tolerated with rare severe adverse events. However, NK cell therapy has not shown significant therapeutic benefit in cancer patients [41, 42]. In this study, we were interested in whether pretreatment with four well-known reprogramming-enhancer small molecules was able to boost the tumor-lytic function of NK cells collected from cancer patients. Our data demonstrate
VPA epigenetically suppresses NK cell cytotoxicity

Figure 6. Epigenetic regulation of NKG2D by VPA. A. Representative FACS histograms of NKG2D expression on the cell surface of NK cells after treatment with VPA. B. Flow cytometry mean fluorescence intensity (MFI) of NKG2D in NK cells after VPA treatment. Data are presented as the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 as compared with the PBS control. C. Real-time RT-PCR quantitation of the NKG2D mRNA transcripts. Data are expressed as relative expression of NKG2D mRNA in VPA-treated NK cells over that of the PBS control cells.
VPA epigenetically suppresses NK cell cytotoxicity

Data are mean ± standard deviation (SD) of NK cells derived from three different donors. Each experiment was run in triplicate. *p < 0.05 as compared with the PBS control. D. Western analysis of NKG2D expression. The abundance of NKG2D proteins was measured by Western blot (top panel) and was quantitated densitometrically over that of β-ACTIN (bottom panel).

that the tumor-lytic activity of NK cells is not significantly affected by DNA demethylating agent 5-AzaC, antioxidant nutrient Vit-C, and thyroid hormone T3. However, treatment with VPA, a histone deacetylase inhibitor that usually activates downstream target genes, dramatically cripples the tumor-lytic function of NK cells in a dose-dependent manner.

VPA is a FDA-approved anti-epileptic agent. VPA and other histone deacetylase inhibitors (HDACi) have been tested for their anticancer activity in clinical trials [43]. The antitumoral activity of HDACi is ascribed to their direct effects on tumor cells by multiple pathways, including the induction of phenotype changes, growth arrest, apoptosis or differentiation, angiogenesis inhibition, effects on DNA repair, mitosis, autophagy, and the misfolded protein response [44, 45], probably through the reactivation of epigenetically silenced tumor suppressor genes. Currently, most of studies, particularly those conducted in vitro, have been focused on the effect of HDACi on tumor target cells. Their activity on aspects of the innate immune system, such as NK cells as reported in this study, has been largely neglected. Data from this study may suggest a detrimental activity of HDACi on cellular immune system against cancer. Thus, it would be of great benefit if the sup-
VPA epigenetically suppresses NK cell cytotoxicity

pressive activity of HDACi on NK cells can be avoided. When screening for new HDACi drug candidates, it should be necessary to determine if the drugs suppress NK cell activity. An HDACi that lacks the NK cell suppressive activity may have enhanced therapeutic potential.

The suppression of NK cell cytotoxicity by HDACi has also been noted in other tumor models. In an orthotopic glioblastoma mouse model treated with oncolytic herpes simplex virus therapy, Alvarez-Breckenridge et al. [46] found that VPA exhibited an immunosuppressive effect on NK cells, in parallel with downregulation of granzyme B and perforin. VPA decreased IFN-γ expression of cytokine-pre-treated NK cells through the STAT5 and T-bet pathway. In leukemic cells, Ogbomo et al. [47] observed that two HDACi (suberoylanilide hydroxamic acid and VPA) at therapeutic concentrations inhibited cytotoxicity of IL-2-activated NK cells. The inhibition was associated with the decreased NK cell activating receptors Nkp46 and Nkp30 as well as impaired granule exocytosis, probably through the inhibition of NFkappaB activation. Similarly, Rossi et al. [48] reported that three HDACi (trichostatin A, VPA, and sodium butyrate) suppressed NK cell-mediated cytotoxicity against K562 cells through inhibition of nuclear mobilization of NF-xB p50. In patients with cutaneous T cell lymphoma, Kelly-Sell et al. [49] observed a decline in their NK cell cytolytic function and the ability of their DCs to be activated or produce IL-12 following the treatment with three cycles of HDACi Romidepsin.

Interestingly, the beneficial effects have been reported for other HDACi. Treatment of peripheral blood mononuclear cells with Chidamide, a new HDACi of the benzamide class currently under clinical development in cancer indications, led to enhanced lysis of K562 tumor cells, in association with induction of NK cell receptors (CD16, NKG2D, and KLRG1), cytotoxic enzymes (granzyme and perforin), and molecules important for apoptosis (FASLG) [50]. Entinostat, a second benzamide-derivative narrow-spectrum HDACi, induced dose- and time-dependent increase expression of NKG2D in primary human NK cells. Entinostat pretreatment of NK cells enhanced cytotoxicity in vitro, which was reversed by NKG2D blockade [51]. It would of great interest to examine whether these two HDACi that lack the NK suppression activity have a superior therapeutic potential in the treatment of tumor patients.

Currently, it is not clear as how VPA attenuates the antitumor activity of NK cells. Using PI+/Annexin-V− staining, we found that VPA enhanced apoptosis in a small fraction of treated NK cells (7.35%, 8.38%, 10.6%, 13.1% in 0.5-4.0 mM VPA groups vs 5.15% in the PBS control group). Clearly, mechanisms other than apoptosis may be associated with the detrimental effect of VAP on cytotoxicity of NK cells. Programmed cell death protein 1 (PD1), a member of the CD28 family of receptors, is activated on the surface of antigen-activated and -exhausted T cells, contributing to T-cell immune dysfunction and suppression of antitumor immunity by virtue of PDL1 interaction [52, 53]. Expression of PD1 is associated with T-cell apoptosis, increased proliferative potential of tumor cells, and resistance to therapies. Treatment using antibodies that block the interaction of PD1 with its ligand, PD-L1 restores the function of exhausted T cells and enhances T-cell responses [54, 55]. Monoclonal antibodies that target the PD1/PD-L1 pathway to boost the immune system, known as immune checkpoint blockade, are being developed for the treatment of several malignancies [56, 57], including metastatic melanoma, and advanced non-small cell lung cancer. Interestingly, we demonstrate in this study that treatment of VPA induces similar dysfunction in NK cells. In parallel, the PD1/PD-L1 pathway is activated in the VPA-treated cells. Our study links the role of the PD-1 inhibitory pathway to VPA-mediated suppression of NK cell cytotoxicity. Future studies are needed to examine if inhibition of the PD1/PD-L1 pathway will be able to abolish the VPA-mediated suppression of NK cell function.

NK cells can mediate cytotoxicity by releasing cytotoxic granules upon interaction with target cells. This cytotoxicity requires the activation of NKG2D, a unique activating receptor of cytotoxic lymphocyte that functions as a sensory signal to alert the immune system to target tumors [58, 59]. In humans, NKG2D transmits signals by its interaction with the DAP10 adapter subunit. Once activated, NKG2D senses recognition of “induced self” and triggers elimination of transformed cells or pathogen-infected cells [60]. In this study, we found that VPA induced a dose-dependent suppression of NKG2D in NK cells (Figure 6). NKG2D is encod-
ed by a highly conserved gene KLRK1. Expression of NKG2D is tightly regulated at the level of transcription.

Using DNA bisulfite sequencing, we showed that there was only a slight increment in DNA methylation in the CpG islands in the gene promoter. Using chromatin immunoprecipitation assay, however, we noticed a significant increase in H3K9me2 levels at the NKG2D locus in VPA-treated NK cells as compared with untreated NK cells (Figure 7). Di-methylation of lysine 9 of histone H3 (H3K9me2) is widely associated with chromatin compaction [61] and transcriptional silencing of euchromatic genes [62, 63]. Thus, our data suggest that VPA suppresses NKG2D in NK cells through an epigenetic mechanism of inducing H3K9 methylation in the gene promoter.

In summary, we compared the effect of four reprogramming-enhancing molecules on tumor cytotoxicity of NK cells. While T3, vitamin C and 5-azaC did not significantly affect NK cell function, histone deacetylase inhibitor VPA impeded the lytic activity of NK cells against leukemic cells in a dose-dependent manner. Treatment of VPA impaired IFNγ secretion and CD107a degranulation through the activation of the PD-1/PD-L1 pathway and the downregulation of the activating receptor NKG2D by inducing histone K9 hypermethylation and DNA methylation in the gene promoter.

Acknowledgements

We thank Dr. Andrew R. Hoffman at Stanford University Medical School for editing the manuscript. This work was supported by California Institute of Regenerative Medicine (CIRM) grant (RT2-01942), Jilin International Collaboration Grant (#20120720), the National Natural Science Foundation of China grant (#81272294, #31430021) to J.F.H; the National Natural Science Foundation of China grant (#81071920, #81372835) and Jilin Science and Technique Program grant (11GG003) to W.L.; the grant of Key Project of Chinese Ministry of Education (#311015) to C.J.; and the National Natural Science Foundation of China grant(#31470798) and Provincial Science Fund of Jilin Provincial Department of Finance (20150204027YY) to H.J.

Disclosure of conflict of interest

None.
VPA epigenetically suppresses NK cell cytotoxicity


VPA epigenetically suppresses NK cell cytotoxicity


VPA epigenetically suppresses NK cell cytotoxicity


VPA epigenetically suppresses NK cell cytotoxicity

Epigenetic suppression of the antitumor cytotoxicity of NK cells by histone deacetylase inhibitor valproic acid

Xiumin Shi¹, Min Li¹, Meizi Cui¹, Chao Niu¹, Jianting Xu¹, Lei Zhou¹, Wei Li¹, Yushun Gao³, Weisheng Kong⁴, Jiwei Cui¹, Ji-Fan Hu¹,², Haofan Jin¹

¹Stem Cell and Cancer Center, First Hospital, Jilin University, Changchun, Jilin 130021, China; ²Stanford University Medical School, Palo Alto Veterans Institute for Research, Palo Alto, CA 94304, USA; ³Department of Thoracic Surgical Oncology, Cancer Institute, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100021, China; ⁴BASO Cell Science & Technology Co., Ltd, Zhuai, Guangdong 519015, China

Table S1. PCR Primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td></td>
</tr>
<tr>
<td>β-ACTIN forward:</td>
<td>AGATCAAGATCATTTGCTCCCATGA</td>
</tr>
<tr>
<td>β-ACTIN reverse:</td>
<td>ATACTCTGTGGCTGATCCATCCATC</td>
</tr>
<tr>
<td>NKG2D forward:</td>
<td>CACAGCTGGAGATGAGTGA</td>
</tr>
<tr>
<td>NKG2D reverse:</td>
<td>TCGTCAAGGGAATTGTGA</td>
</tr>
<tr>
<td>PD-1 forward:</td>
<td>CGTGCCCTATCCACTCCTCA</td>
</tr>
<tr>
<td>PD-1 reverse:</td>
<td>ATCCCTTTGCCCAGCCACTC</td>
</tr>
<tr>
<td>PD-L1 forward:</td>
<td>AAATGGAAACCTGGGAAAGC</td>
</tr>
<tr>
<td>PD-L1 reverse:</td>
<td>GATGAGCCCCTACGGCATTT</td>
</tr>
<tr>
<td>DNA methylation</td>
<td></td>
</tr>
<tr>
<td>NKG2D Amp-1 forward:</td>
<td>GGTTGAGGTTAGGAGTTATTTTGAGTT</td>
</tr>
<tr>
<td>NKG2D Amp-1 reverse:</td>
<td>CACTCTATTATCCAACTAAAATAC</td>
</tr>
<tr>
<td>NKG2D Amp-2 forward:</td>
<td>GATGTTTAGGAAATTTTTGAGG</td>
</tr>
<tr>
<td>NKG2D Amp-2 reverse:</td>
<td>ATTACCAAATCTAAATTCTAATAAA</td>
</tr>
<tr>
<td>NKG2D Amp-3 forward:</td>
<td>TTGGAAATATTTGATTTTAGTTAA</td>
</tr>
<tr>
<td>NKG2D Amp-3 reverse:</td>
<td>AAAATAACAACTTCCATAAAAACCAAC</td>
</tr>
<tr>
<td>CHIP assay</td>
<td></td>
</tr>
<tr>
<td>NKG2D Amp1 forward:</td>
<td>GCACAGGGGAAAGTTTCTG</td>
</tr>
<tr>
<td>NKG2D Amp1 reverse:</td>
<td>AGTGTACACGGAGAGGAA</td>
</tr>
<tr>
<td>NKG2D Amp2 forward:</td>
<td>GGGTCAAGAAGCAATAAGC</td>
</tr>
<tr>
<td>NKG2D Amp2 reverse:</td>
<td>GCAGAAACTTTCCCTG</td>
</tr>
</tbody>
</table>

Figure S1. Modification of the NK cells by reprogramming molecules in the absence of cytokines NK cells were treated with (T3 20 μM, vitamin C 1 mM, azacitidine 2 μM, VPA 1 mM) in the absence of cytokines (IL2) in the medium. Twenty-four hours after treatment, cells were tested for the specific lysis against K562 cells at the indicated effector:target (E:T) ratios. *p < 0.05 between the treated and control NK cells.