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Original Article

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

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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 immuno-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 CAR-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% CO_2.

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 2×10^6 cells/ml in six-well plates in a humidified atmosphere with 5% CO_2 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

Table 1. Characteristics of the patients with lung cancers

No.	Gender	Age (yrs)	Diagnosis	Stage*
1	M	56	Lung cancer (adenocarcinoma)	T4N2M0, IA
2	M	54	Lung cancer (adenocarcinoma)	T2N0M0, IB
3	M	64	Lung cancer (adenocarcinoma)	T2N0M0, IB
4	M	48	Lung cancer (adenocarcinoma)	T2N0M0, IB
5	M	68	Lung cancer (adenocarcinoma)	T2N3M0,IIIB
6	M	59	Lung cancer (adenocarcinoma)	T1N0M0, IIIB
7	F	78	Lung cancer (adenocarcinoma)	T3N3M1, IV
8	F	67	Lung cancer (adenocarcinoma)	T4N2M1, IV
9	M	71	Lung cancer (adenocarcinoma)	T2N3M1, IV
10	F	39	Lung cancer (adenocarcinoma)	T4N3M1, IV
11	F	57	Lung cancer (squamous carcinoma)	T4N2M0, IIA
12	M	80	Lung cancer (squamous carcinoma)	T2N2M0, IIB
13	M	74	Lung cancer (squamous carcinoma)	T2N1M0, IIIA
14	M	71	Lung cancer (squamous carcinoma)	T1N1M0, IIIB
15	F	46	Lung cancer (large cell carcinoma)	T1N1M0, IIB
16	F	32	Lung cancer (large cell carcinoma)	T3N0M0, IIIA
17	M	51	Lung cancer (small cell)	Limited stage
18	F	38	Lung cancer (small cell)	Limited stage
19	M	48	Lung cancer (small cell)	Limited stage
20	M	58	Lung cancer (small cell)	Extensive stage
21	M	69	Lung cancer (small cell)	Extensive stage
22	M	52	Lung cancer (small cell)	Extensive stage
23	F	71	Lung cancer (small cell)	Extensive stage
24	F	45	Lung cancer (small cell)	Extensive stage

*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].

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 [(experimental release-spontaneous release)/(maximum release-spontaneous release)]×100.

Flow cytometry analysis

Surface expression of *NKG2D* and CD107a was analyzed with a FACSCalibur 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 2×10^6 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 $2^{-(\Delta Ct)}$ 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,

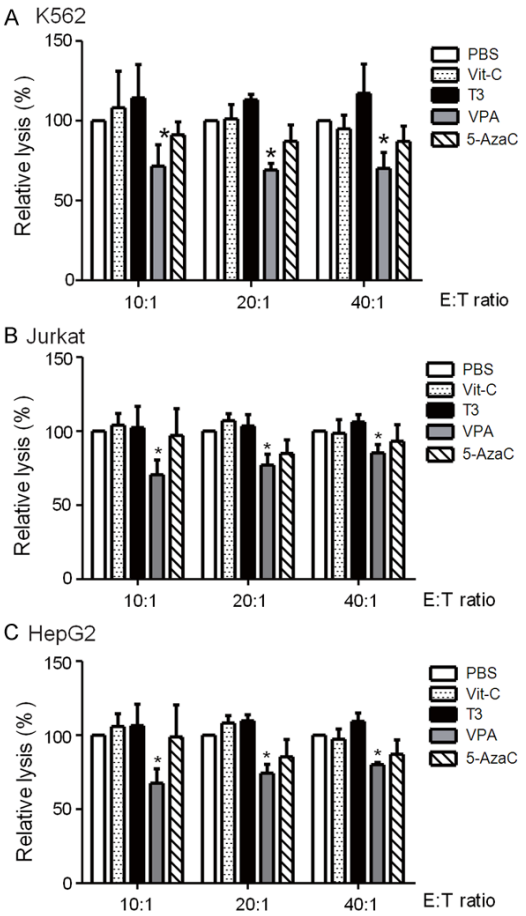

Figure 1. Modification of the cytotoxicity of NK cells by reprogramming molecules. NK cells were treated with reprogramming molecules for 24 h (T3 20 μM, vitamin C 1 mM, azacytidine 2 μM, VPA 1 mM) and tested for the specific lysis against tumors at the indicated effctor:target (E:T) ratios. A. K562 cells; B. Jurkat cells; C. HepG2 cells. *p < 0.05 between the treated and control NK cells.

0.25% deoxycholate, 1% Triton X-100 and 0.5 M NaCl). Protein concentrations were determined using Compatibility Chart For BCA Kit (Beyotime, Wuhan, China). Equal aliquots of protein sample (30 μg/lane) were separated by 12% SDS-PAGE gel and transferred onto PVDF membranes. The blot was then incubated 1 h with blocking buffer containing 0.1% Tween 20 and 5% nonfat dry milk. The membrane were incubated with primary antibodies *NKG2D* (SAB2500697, Sigma, MO) with a dilution of 1:1700 and then with donkey anti-goat IgG-HRP (Boster, Wuhan, China). The STAT5 pathway was examined using phospho-stat5 antibody (#4322, Tyr694-D47E7 XP® Rabbit mAb, Cell Signaling Technology, MA). The ECL detection system was used to visualize the proteins. Band intensity of western blots was determined by densitometry with ImageJ.

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 [Ct (BOUND)-(Ct(UNBOUND)-$\log_2$ (UNBOUND DILUTION FACTOR))]×100.

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 tatistically 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].

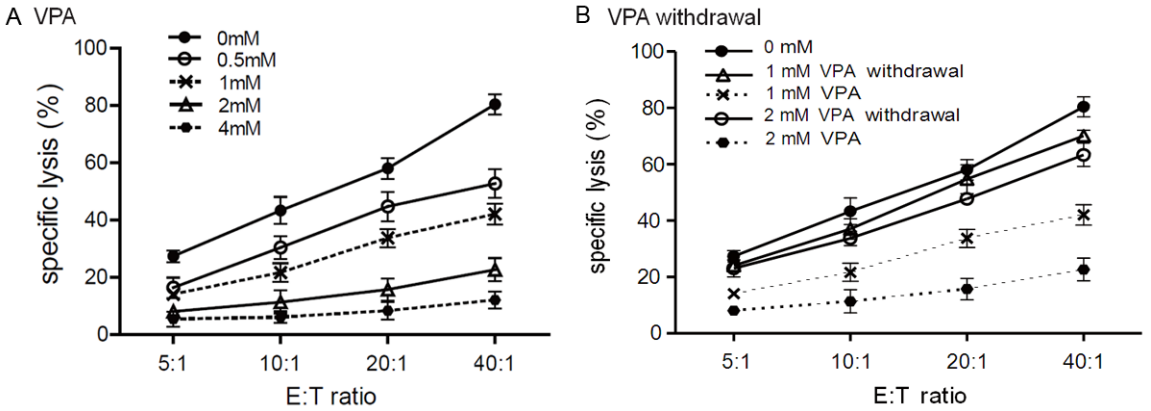

Figure 2. VPA dose-dependently suppresses the cytotoxicity of NK cells. A. Epigenetic suppression of NK cell cytotoxicity by VPA. NK cells were treated with VPA (0.5 mM, 1 mM, 2 mM, 4 mM) for 24 hrs and the cytolytic activity against K562 cells was assessed at the indicated effctor:target (E:T) ratios. B. Reversible suppression of NK cells by VPA. NK cells were treated with VPA (1 mM, 2 mM) for 24 hrs and were switched to normal medium in the absence of VPA. After washout for 48 h, NK cells were examined for the cytotoxicity against K562 cells. * $p < 0.05$ between VPA treated NK cells and after withdrawal of VPA.

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 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

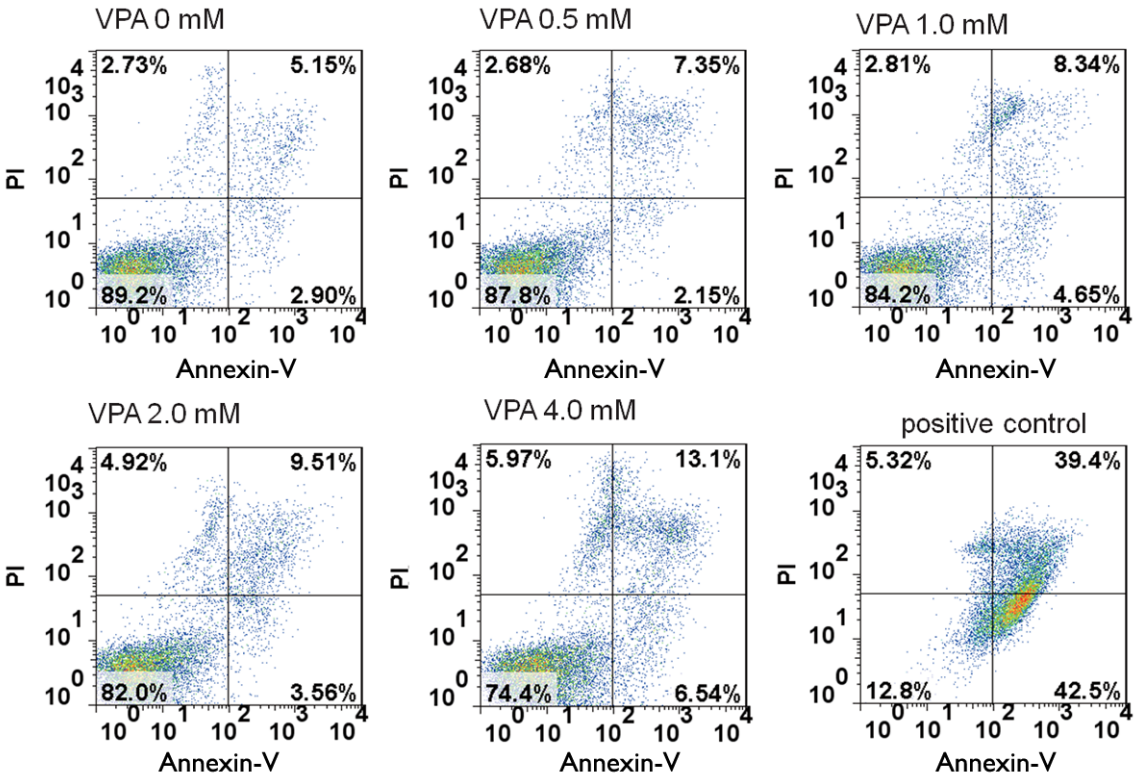

Figure 3. VPA induced apoptosis in NK cells. NK cells were treated with 0.5 mM, 1 mM, 2 mM, and 4 mM of VPA. After PI/Annexin-V staining, cell viability and apoptosis were measured by FACS.

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

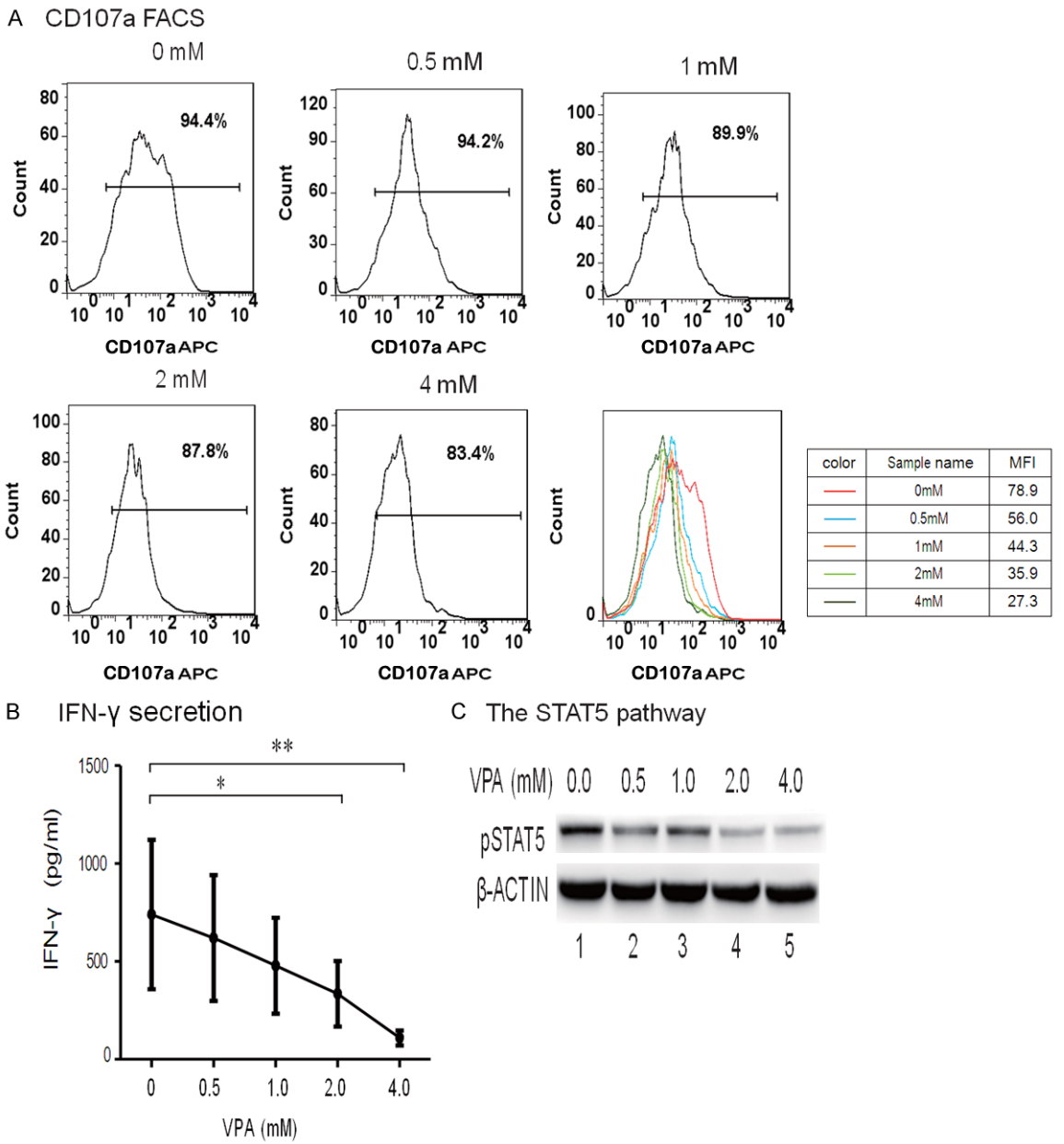

Figure 4. VPA downregulates CD107a and impairs IFN-γ secretion. A. VPA downregulated CD107a expression in NK cells. After treatment with VPA, NK cells were evaluated by FACS for the percentage of CD107a+ cells and the mean fluorescence intensity (MFI). B. VPA decreased IFN-γ secretion. NK cells were treated with VPA and the supernatants were collected for the measurement of IFN-γ using ELISA Kit. The graph shows the mean ± SD obtained from six independent donors. *$p < 0.05$, **$p < 0.01$ as compared with the PBS control. C. VPA downregulated the STAT5 pathway. The activity of the STAT5 pathway was measured by Western blot using antibody against phospho-stat5.

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-

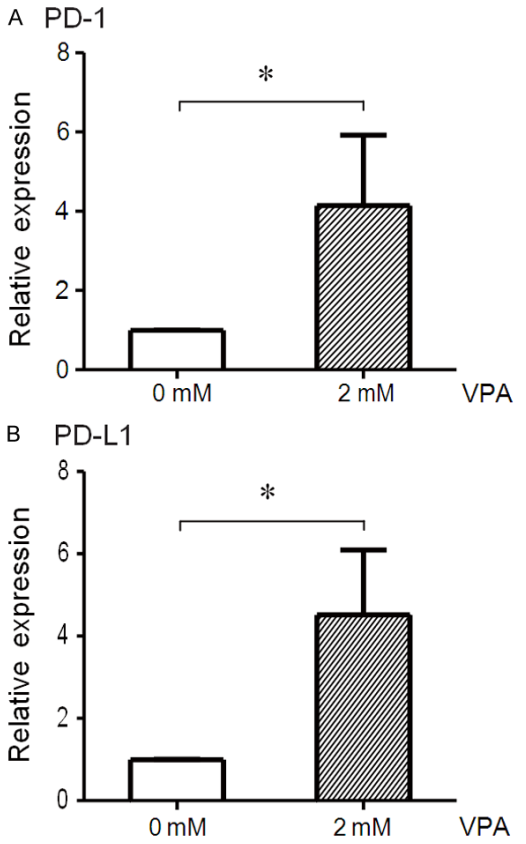

Figure 5. VPA activates the PD-1 and PD-L1 pathway in NK cells. NK cells were treated with VPA (0.5 mM, 1 mM, 2 mM, 4 mM for 24 h) and total RNA was extracted for the expression analysis of PD-1 and PD-L1 by quantitative PCR. *$p < 0.01$ as compared with the PBS control.

ral-killer group 2, member D), a unique activating receptor of cytotoxic lymphocytes. We determined the cell-surface expression of *NKG2D* using flow cytometry and analyzed how *NKG2D* expression was modified by VPA in NK cells. There was a significant dose-dependent downregulation of *NKG2D* expression on the cell surface, as measured by the percentage of NKG2D positive cells (**Figure 6A**) and the Mean Fluorescence Intensity (MFI) (**Figure 6B**).

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

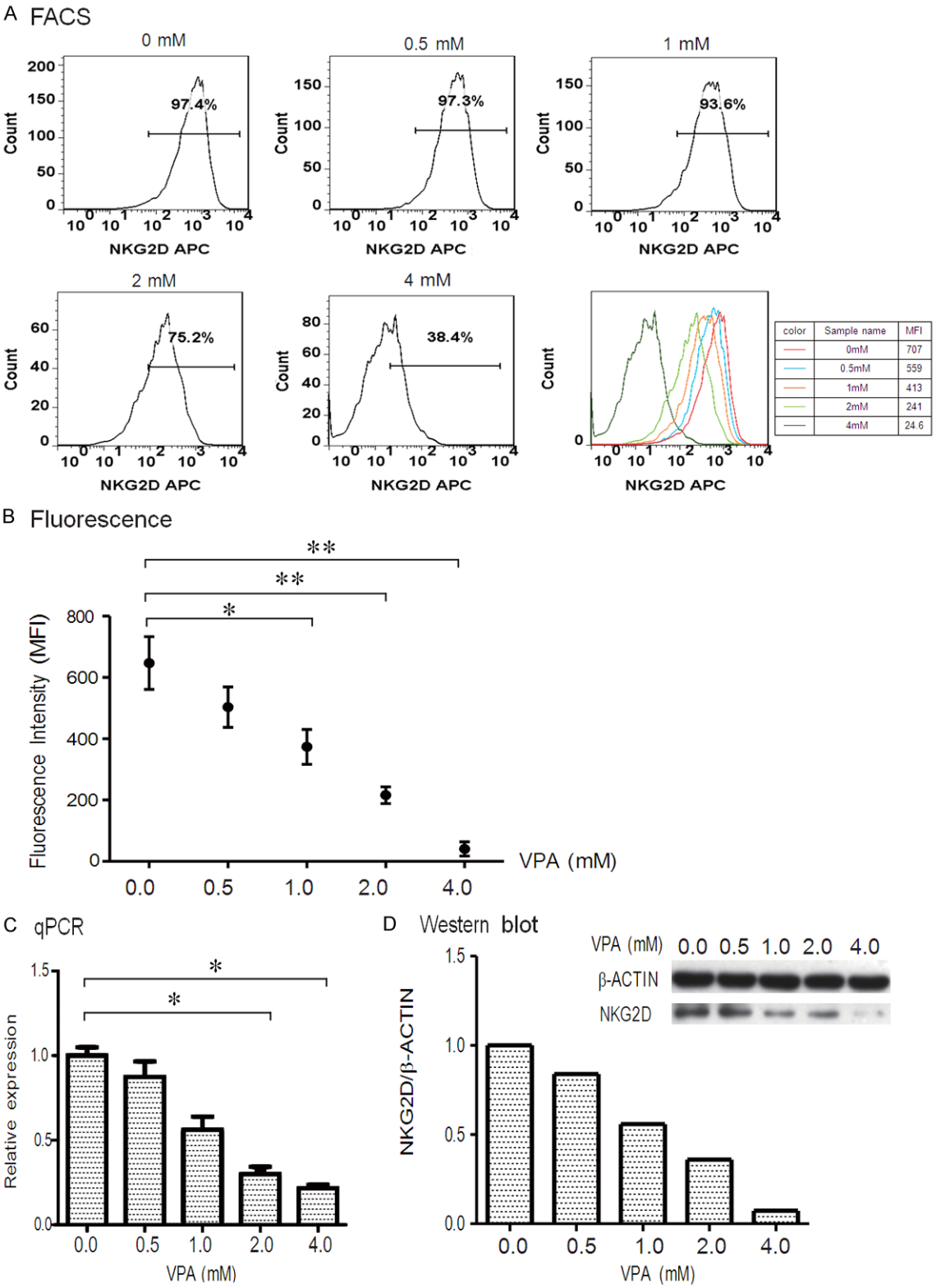

Figure 6. Epigenetic regulation of NKG2D by VPA. A. Representative FACS histograms of NKG2D expression on the cell surface of NK cells after treatment with VPA. B. Flow cytometry mean fluorescence intensity (MFI) of NKG2D in NK cells after VPA treatment. Data are presented as the mean ± SD of three independent 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.

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 ß-ACTIN (bottom panel).

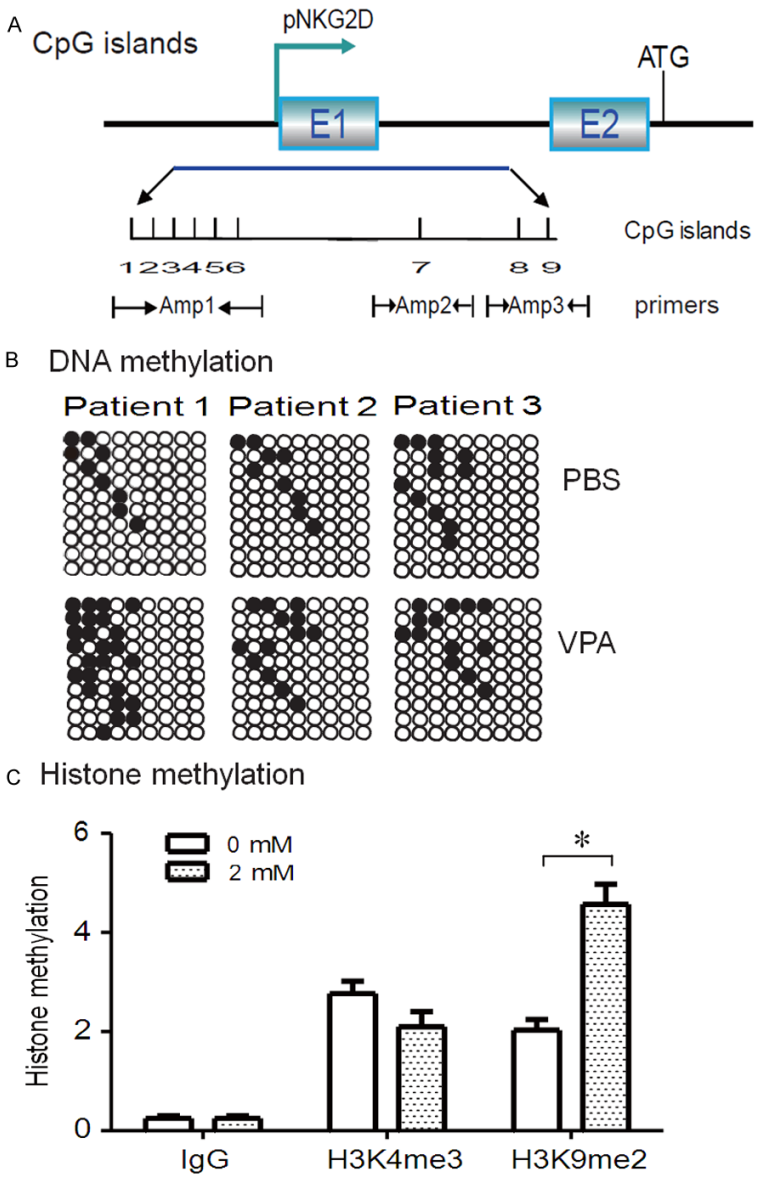

Figure 7. Epigenetic regulation of NKG2D promoter. A. Schematic diagram of CpG islands in the *NKG2D* promoter. Vertical lines: location of CpG islands. B. DNA methylation of the *NKG2D* promoter in NK cells collected from three patients. Genomic DNAs was quantitated by bisulphate sequencing. Open circles: unmethylated CpGs; solid circles: methylated CpGs. C. Histone methylation in the *NKG2D* gene promoter. Levels of histone modifications in the *NKG2D* promoter were measured by ChIP assay using antibodies specific for H3K4me3 and H3K9me2 in NK cells treated with VPA. Normal rabbit IgG was used as a negative control. Enrichment of *NKG2D*-specific DNA sequences was measured by quantitative PCR. Data are shown from three independent experiments and each qRT-PCR was performed in triplicate. *p < 0.05 between the VPA-treated and the PBS control NK cells.

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 antiepileptic 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-

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-κB 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.

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Disclosure of conflict of interest

None.

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References

[1] Campbell KS and Hasegawa J. Natural killer cell biology: an update and future directions. J Allergy Clin Immunol 2013; 132: 536-544.

[2] Childs RW and Carlsten M. Therapeutic approaches to enhance natural killer cell cytotoxicity against cancer: the force awakens. Nat Rev Drug Discov 2015; 14: 487-498.

[3] Klingemann H. Challenges of cancer therapy with natural killer cells. Cytotherapy 2015; 17: 245-249.

[4] Tian Z, Gershwin ME and Zhang C. Regulatory NK cells in autoimmune disease. J Autoimmun 2012; 39: 206-215.

[5] Hodny Z, Hubackova S and Bartek J. Cytokine-induced 'bystander' senescence in DDR and immuno-surveillance. Oncotarget 2013; 4: 1552-1553.

[6] Locatelli F, Moretta F, Brescia L and Merli P. Natural killer cells in the treatment of high-risk acute leukaemia. Semin Immunol 2014; 26: 173-179.

[7] Jewett A and Tseng HC. Tumor induced inactivation of natural killer cell cytotoxic function; implication in growth, expansion and differentiation of cancer stem cells. J Cancer 2011; 2: 443-457.

[8] Lim O, Jung MY, Hwang YK and Shin EC. Present and Future of Allogeneic Natural Killer Cell Therapy. Front Immunol 2015; 6: 286.

[9] Tasian SK and Gardner RA. CD19-redirected chimeric antigen receptor-modified T cells: a promising immunotherapy for children and adults with B-cell acute lymphoblastic leukemia (ALL). Ther Adv Hematol 2015; 6: 228-241.

[10] Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL and June CH. Chimeric antigen receptor- modified T cells for acute lymphoid leukemia. N Engl J Med 2013; 368: 1509-1518.

[11] Kochenderfer JN, Dudley ME, Carpenter RO, Kassim SH, Rose JJ, Telford WG, Hakim FT, Halverson DC, Fowler DH, Hardy NM, Mato AR,

Hickstein DD, Gea-Banacloche JC, Pavletic SZ, Sportes C, Maric I, Feldman SA, Hansen BG, Wilder JS, Blacklock-Schuver B, Jena B, Bishop MR, Gress RE and Rosenberg SA. Donor-derived CD19- targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. Blood 2013; 122: 4129-4139.

[12] Magee MS and Snook AE. Challenges to chimeric antigen receptor (CAR)-T cell therapy for cancer. Discov Med 2014; 18: 265-271.

[13] Yasmin R, Siraj S, Hassan A, Khan AR, Abbasi R and Ahmad N. Epigenetic regulation of inflammatory cytokines and associated genes in human malignancies. Mediators Inflamm 2015; 2015: 201703.

[14] Hasim A, Abudula M, Aimiduo R, Ma JQ, Jiao Z, Akula G, Wang T and Abudula A. Post-transcriptional and epigenetic regulation of antigen processing machinery (APM) components and HLA-I in cervical cancers from Uighur women. PLoS One 2012; 7: e44952.

[15] Tertipis N, Haeggblom L, Grun N, Nordfors C, Nasman A, Dalianis T and Ramqvist T. Reduced Expression of the Antigen Processing Machinery Components TAP2, LMP2, and LMP7 in Tonsillar and Base of Tongue Cancer and Implications for Clinical Outcome. Transl Oncol 2015; 8: 10-17.

[16] Wang LX, Mei ZY, Zhou JH, Yao YS, Li YH, Xu YH, Li JX, Gao XN, Zhou MH, Jiang MM, Gao L, Ding Y, Lu XC, Shi JL, Luo XF, Wang J, Wang LL, Qu C, Bai XF and Yu L. Low dose decitabine treatment induces CD80 expression in cancer cells and stimulates tumor specific cytotoxic T lymphocyte responses. PLoS One 2013; 8: e62924.

[17] Dubovsky JA, Powers JJ, Gao Y, Mariusso LF, Sotomayor EM and Pinilla-Ibarz JA. Epigenetic repolarization of T lymphocytes from chronic lymphocytic leukemia patients using 5-aza-2'-deoxycytidine. Leuk Res 2011; 35: 1193-1199.

[18] Wernig M, Lengner CJ, Hanna J, Lodato MA, Steine E, Foreman R, Staerk J, Markoulaki S and Jaenisch R. A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nat Biotechnol 2008; 26: 916-924.

[19] Esteban MA, Wang T, Qin B, Yang J, Qin D, Cai J, Li W, Weng Z, Chen J, Ni S, Chen K, Li Y, Liu X, Xu J, Zhang S, Li F, He W, Labuda K, Song Y, Peterbauer A, Wolbank S, Redl H, Zhong M, Cai D, Zeng L and Pei D. Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. Cell Stem Cell 2010; 6: 71-79.

[20] Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W and Melton DA. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 2008; 26: 1269-1275.

[21] Chen M, Zhang H, Wu J, Xu L, Xu D, Sun J, He Y, Zhou X, Wang Z, Wu L, Xu S, Wang J, Jiang S, Hoffman AR, Hu X, Hu J and Li T. Promotion of the induction of cell pluripotency through metabolic remodeling by thyroid hormone triiodothyronine- activated PI3K/AKT signal pathway. Biomaterials 2012; 33: 5514-5523.

[22] Cui J, Li L, Wang C, Jin H, Yao C, Wang Y, Li D, Tian H, Niu C, Wang G, Han W, Xu J, Chen J and Li W. Combined cellular immunotherapy and chemotherapy improves clinical outcome in patients with gastric carcinoma. Cytotherapy 2015; 17: 979-988.

[23] Kang L, Sun J, Wen X, Cui J, Wang G, Hoffman AR, Hu JF and Li W. Aberrant allele- switch imprinting of a novel IGF1R intragenic antisense non-coding RNA in breast cancers. Eur J Cancer 2015; 51: 260-270.

[24] Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA, Rami-Porta R, Postmus PE, Rusch V, Sobin L; International Association for the Study of Lung Cancer International Staging Committee; Participating Institutions. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol 2007; 2: 706-714.

[25] Pallis AG, Shepherd FA, Lacombe D and Gridelli C. Treatment of small-cell lung cancer in elderly patients. Cancer 2010; 116: 1192-1200.

[26] Neri S, Mariani E, Meneghetti A, Cattini L and Facchini A. Calcein-acetyoxymethyl cytotoxicity assay: standardization of a method allowing additional analyses on recovered effector cells and supernatants. Clin Diagn Lab Immunol 2001; 8: 1131-1135.

[27] Sun J, Li W, Sun Y, Yu D, Wen X, Wang H, Cui J, Wang G, Hoffman AR and Hu JF. A novel antisense long noncoding RNA within the IGF1R gene locus is imprinted in hematopoietic malignancies. Nucleic Acids Res 2014; 42: 9588-9601.

[28] Zhang H, Niu B, Hu JF, Wang H, Ling J, Qian G, Ge S and Hoffman AR. Interruption of intrachromosomal looping by CTCF decoy proteins abrogates genomic imprinting of human insulin-like growth factor II. J Cell Biol 2011; 193: 475-487.

[29] Li T, Hu JF, Qiu X, Ling J, Chen H, Wang S, Hou A, Vu TH and Hoffman AR. CTCF regulates allelic expression of Igf2 by orchestrating a promoter-polycomb repressive complex-2 intrachromosomal loop. Mol Cell Biol 2008; 28: 6473-6482.

[30] Zhang S, Zhong B, Chen M, Yang G, Li Y, Wang H, Wang G, Li W, Cui J, Hoffman AR and Hu J. Epigenetic reprogramming reverses the malignant epigenotype of the MMP/TIMP axis genes in tumor cells. Int J Cancer 2014; 134: 1583-1594.

[31] Li T, Chen H, Li W, Cui J, Wang G, Hu X, Hoffman AR and Hu J. Promoter histone H3K27 methylation in the control of IGF2 imprinting in human tumor cell lines. Hum Mol Genet 2014; 23: 117-128.

[32] Zhang H, Jiao W, Sun L, Fan J, Chen M, Wang H, Xu X, Shen A, Li T, Niu B, Ge S, Li W, Cui J, Wang G, Sun J, Fan X, Hu X, Mrsny RJ, Hoffman AR and Hu JF. Intrachromosomal Looping Is Required for Activation of Endogenous Pluripotency Genes during Reprogramming. Cell Stem Cell 2013; 13: 30-35.

[33] Serrano A, Castro-Vega I and Redondo M. Role of gene methylation in antitumor immune response: implication for tumor progression. Cancers (Basel) 2011; 3: 1672-1690.

[34] Sola C, Andre P, Lemmers C, Fuseri N, Bonnafous C, Blery M, Wagtmann NR, Romagne F, Vivier E and Ugolini S. Genetic and antibody-mediated reprogramming of natural killer cell missing-self recognition in vivo. Proc Natl Acad Sci U S A 2009; 106: 12879-12884.

[35] Payne KK, Zoon CK, Wan W, Marlar K, Keim RC, Kenari MN, Kazim AL, Bear HD and Manjili MH. Peripheral blood mononuclear cells of patients with breast cancer can be reprogrammed to enhance anti-HER-2/neu reactivity and overcome myeloid-derived suppressor cells. Breast Cancer Res Treat 2013; 142: 45-57.

[36] Chen J, Guo L, Zhang L, Wu H, Yang J, Liu H, Wang X, Hu X, Gu T, Zhou Z, Liu J, Liu J, Wu H, Mao SQ, Mo K, Li Y, Lai K, Qi J, Yao H, Pan G, Xu GL and Pei D. Vitamin C modulates TET1 function during somatic cell reprogramming. Nat Genet 2013; 45: 1504-1509.

[37] Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M and Koup RA. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 2003; 281: 65-78.

[38] Alter G, Malenfant JM and Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004; 294: 15-22.

[39] Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012; 12: 252-264.

[40] Muenst S, Soysal SD, Tzankov A and Hoeller S. The PD-1/PD-L1 pathway: biological background and clinical relevance of an emerging treatment target in immunotherapy. Expert Opin Ther Targets 2015; 19: 201-211.

[41] Sakamoto N, Ishikawa T, Kokura S, Okayama T, Oka K, Ideno M, Sakai F, Kato A, Tanabe M, Enoki T, Mineno J, Naito Y, Itoh Y and Yoshikawa T. Phase I clinical trial of autologous NK cell therapy using novel expansion method in patients with advanced digestive cancer. J Transl Med 2015; 13: 277.

[42] Parkhurst MR, Riley JP, Dudley ME and Rosenberg SA. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. Clin Cancer Res 2011; 17: 6287-6297.

[43] Slingerland M, Guchelaar HJ and Gelderblom H. Histone deacetylase inhibitors: an overview of the clinical studies in solid tumors. Anticancer Drugs 2014; 25: 140-149.

[44] Juo YY, Gong XJ, Mishra A, Cui X, Baylin SB, Azad NS and Ahuja N. Epigenetic therapy for solid tumors: from bench science to clinical trials. Epigenomics 2015; 7: 215-235.

[45] Grant S and Dai Y. Histone deacetylase inhibitors and rational combination therapies. Adv Cancer Res 2012; 116: 199-237.

[46] Alvarez-Breckenridge CA, Yu J, Price R, Wei M, Wang Y, Nowicki MO, Ha YP, Bergin S, Hwang C, Fernandez SA, Kaur B, Caligiuri MA and Chiocca EA. The histone deacetylase inhibitor valproic acid lessens NK cell action against oncolytic virus-infected glioblastoma cells by inhibition of STAT5/T-BET signaling and generation of gamma interferon. J Virol 2012; 86: 4566-4577.

[47] Ogbomo H, Michaelis M, Kreuter J, Doerr HW and Cinatl J Jr. Histone deacetylase inhibitors suppress natural killer cell cytolytic activity. FEBS Lett 2007; 581: 1317-1322.

[48] Rossi LE, Avila DE, Spallanzani RG, Ziblat A, Fuertes MB, Lapyckyj L, Croci DO, Rabinovich GA, Domaica CI and Zwirner NW. Histone deacetylase inhibitors impair NK cell viability and effector functions through inhibition of activation and receptor expression. J Leukoc Biol 2012; 91: 321-331.

[49] Kelly-Sell MJ, Kim YH, Straus S, Benoit B, Harrison C, Sutherland K, Armstrong R, Weng WK, Showe LC, Wysocka M and Rook AH. The histone deacetylase inhibitor, romidepsin, suppresses cellular immune functions of cutaneous T-cell lymphoma patients. Am J Hematol 2012; 87: 354-360.

[50] Ning ZQ, Li ZB, Newman MJ, Shan S, Wang XH, Pan DS, Zhang J, Dong M, Du X and Lu XP. Chidamide (CS055/HBI-8000): a new histone deacetylase inhibitor of the benzamide class with antitumor activity and the ability to enhance immune cell-mediated tumor cell cytotoxicity. Cancer Chemother Pharmacol 2012; 69: 901-909.

[51] Zhu S, Denman CJ, Cobanoglu ZS, Kiany S, Lau CC, Gottschalk SM, Hughes DP, Kleinerman ES and Lee DA. The narrow-spectrum HDAC inhibitor entinostat enhances NKG2D expression without NK cell toxicity, leading to enhanced recognition of cancer cells. Pharm Res 2015; 32: 779-792.

[52] Ray A, Das DS, Song Y, Richardson P, Munshi NC, Chauhan D and Anderson KC. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions withT cells, natural killer cells and multiple myeloma cells. Leukemia 2015; 29: 1441-1444.

[53] Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M and Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. J Exp Med 2012; 209: 1201-1217.

[54] Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ and Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 2006; 439: 682-687.

[55] Golden-Mason L, Palmer B, Klarquist J, Mengshol JA, Castelblanco N and Rosen HR. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. J Virol 2007; 81: 9249-9258.

[56] Niezgoda A, Niezgoda P and Czajkowski R. Novel Approaches to Treatment of Advanced Melanoma: A Review on Targeted Therapy and Immunotherapy. Biomed Res Int 2015; 2015: 851387.

[57] Merelli B, Massi D, Cattaneo L and Mandala M. Targeting the PD1/PD-L1 axis in melanoma: biological rationale, clinical challenges and opportunities. Crit Rev Oncol Hematol 2014; 89: 140-165.

[58] Carapito R and Bahram S. Genetics, genomics, and evolutionary biology of NKG2D ligands. Immunol Rev 2015; 267: 88-116.

[59] Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, Eldridge P, Leung WH and Campana D. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Res 2009; 69: 4010-4017.

[60] Lanier LL. NKG2D Receptor and Its Ligands in Host Defense. Cancer Immunol Res 2015; 3: 575-582.

[61] Hublitz P, Albert M and Peters AH. Mechanisms of transcriptional repression by histone lysine methylation. Int J Dev Biol 2009; 53: 335-354.

[62] Kimura H. Histone modifications for human epigenome analysis. J Hum Genet 2013; 58: 439-445.

[63] Nakayama J, Rice JC, Strahl BD, Allis CD and Grewal SI. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science 2001; 292: 110-113.

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

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Table S1. PCR Primers used in the study

Primer name	Primer sequence (5'-3')
RT-PCR	
β-ACTIN forward:	AGATCAAGATCATTGCTCCTCCTGA
β-ACTIN reverse:	ATACTCCTGCTTGCTGATCCACATC
NKG2D forward:	CACAGCTGGGAGATGAGTGA
NKG2D reverse:	TCGGTCAAGGGAATTTGAAC
PD-1 forward:	CGTGGCCTATCCACTCCTCA
PD-1 reverse:	ATCCCTTGTCCCAGCCACTC
PD-L1 forward:	AAATGGAACCTGGCGAAAGC
PD-L1 reverse:	GATGAGCCCCTCAGGCATTT
DNA methylation	
NKG2D Amp-1 forward:	GGTTGAGGTAGGAGGATTATTTGAGGTT
NKG2D Amp-1 reverse:	CACTCTTATTATCCAAACTAAAATAC
NKG2D Amp-2 forward:	GATGTTTGAATAGAATTTTTGAGG
NKG2D Amp-2 reverse:	ATTACAACTTCTAATTCTTCAATAAA
NKG2D Amp-3 forward:	TTGGAAATATTTGATTTTAGTTAAA
NKG2D Amp-3 reverse:	AAAATAACAATTCACATAAAACCAAAC
CHIP assay	
NKG2D Amp1 forward:	GCACAGGGGAAAAGTTTCTG
NKG2D Amp1 reverse:	ACGTCTACCGCAGAGAGGAA
NKG2D Amp2 forward:	GGCCAAGAAGCAATAAACGA
NKG2D Amp2 reverse:	GCAGAAACTTTTCCCCTGTG

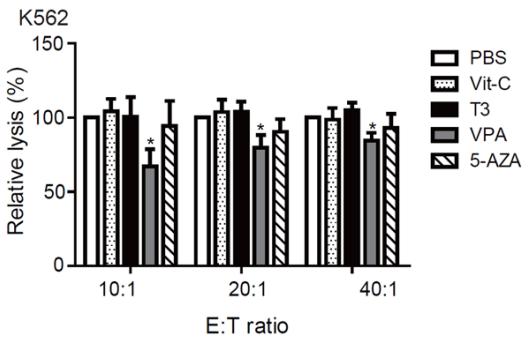

Figure S1. Modification of the NK cells by reprogramming molecules in the absence of cytokines NK cells were treated with (T3 20 μM, vitamin C 1 mM, azacytidine 2 μM, VPA 1 mM) in the absence of cytokines (IL2) in the medium. Twenty-four hours after treatment, cells were tested for the specific lysis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