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*Original article*

# Effect of suberoylanilide hydroxamic acid on peripheral blood mononuclear cell cytotoxicity towards tumor cells in canines

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## Abstract

Suberoylanilide hydroxamic acid (SAHA) is a histone deacetylase inhibitor (HDACi) that suppresses the growth of tumor cells in humans and canines. SAHA reportedly enhances the antitumor activity of human peripheral blood mononuclear cell (PBMC). However, it is unclear whether a similar effect is exerted in canines. The present study focused on the effect of SAHA on the cytotoxicity of IL-2 activated PBMC in three tumor cell lines (CTAC, CIPm, and MCM-N1). The mRNA expression of a ligand for the NKG2D receptor was upregulated in SAHA-treated cell lines. Moreover, the SAHA-treated cell lines, except MCM-N1 demonstrated a significantly higher PBMC cytotoxicity compared to the untreated cell lines. Therefore, the NKG2DL upregulation likely enhanced the interaction of NKG2D-NKG2DL, leading to enhanced cytotoxicity of PBMC. It was also revealed that activated PBMC treated with SAHA significantly attenuated their cytotoxicity toward all the cell lines. Although the NKG2D, NKp46, NKp44, and NKp30 receptors, involved in PBMC cytotoxicity, were presumed to be down-regulated, there was no significant reduction in the mRNA expression of these receptors. This study revealed that SAHA not only sensitizes the canine tumor cells to cytotoxicity due to PBMC activation, but also suppresses the cytotoxicity of PBMC themselves. Therefore, our results highlight the necessity of avoiding this inhibitory action to enhance the antitumor effect of SAHA in canines.

**Key words:** antitumor effect, canines, cytotoxicity, NKG2D receptor, peripheral blood mononuclear cell, suberoylanilide hydroxamic acid

## Introduction

Multimodal treatments to treat tumors comprise effective strategies such as strengthening the immune system. The intensity of peripheral blood mononuclear cell (PBMC) cytotoxicity is correlated with the incidence of tumors (Imai et al. 2000). Therefore, a method that enhances the antitumor activity of the immune cells may prove to be a significant immunological approach.

In PBMC, the natural killer (NK) cells and T cells play an important role in the elimination of tumor and virus-infected cells as immune effector cells in the innate and adaptive immune systems, respectively. NK cells, unlike T cells, do not express specific antigen receptors, and their cytotoxicity is regulated by balancing the signals between the activating and inhibitory cell surface receptors. The cytotoxicity of NK cells to target cells is associated with the receptors triggering the NK response, such as the natural killer group 2D (NKG2D) and natural cytotoxicity receptors (NCRs) including NKp46, NKp44, and NKp30 (Bauer et al. 1999, Moretta et al. 2000). NKG2D and NCR knock-outs have been demonstrated to promote the progression of tumor growth (Guerra et al. 2008, Halftack et al. 2009, Elboim et al. 2010).

Although the expression of NCRs is restricted to the NK cells, NKG2D is expressed not only in the NK cells but also in the  $\alpha/\beta^+$  CD8 and  $\gamma/\delta^+$  T cells (Bauer et al. 1999). The ligands for NKG2D have been well investigated in humans and mice. The NKG2D ligand (NKG2DL) is constitutively expressed in tumor cells, with an upregulated expression in response to cellular stress and viral/bacterial infection (Groh et al. 1996, 1999, 2001, Li et al. 2001, Cosman et al. 2001, Steinle et al. 2001). It has been suggested that sufficient levels of NKG2D-NKG2DL interactions abrogate the inhibitory signals transmitted by MHC class I recognition, sensitizing the NK cells to cytotoxicity (Bauer et al. 1999, Cerwenka et al. 2001, Diefenbach et al. 2001). Therefore, the NKG2D-NKG2DL interaction plays a critical role in tumor rejection and surveillance, and the upregulation of NKG2DL expression on target cells appears to exert an effective antitumor effect (Lanier 2001). However, the expression of NKG2DL in canine tumors has not been extensively studied.

In recent years, the histone deacetylase inhibitors (HDACi), which is an epigenetic drug, has been intensively studied for application as an antitumor drug (McLaughlin et al. 2003). HDACi induces cellular chromatin remodeling by increasing the acetylation of DNA-related histone proteins, leading to cell cycle arrest, differentiation, and apoptosis of the tumor cells. These effects are restricted to the tumor cells without affecting the non-malignant cells (Johnstone 2002).

Interestingly, HDACi has been shown to have a unique effect on upregulating the NKG2DL expression. This effect enhances the NKG2D-NKG2DL interaction, sensitizing the tumor cells to immune effector cell cytotoxicity (Cinatl et al. 1996, Armeanu et al. 2005, Skov et al. 2005, Schmudde et al. 2008). Therefore, HDACi may be a beneficial agent for enhancing the immune system.

Suberoylanilide hydroxamic acid (SAHA) is a broad-spectrum HDACi approved by the US Food and Drug Administration as a drug for cutaneous T-cell lymphoma in humans (Marks and Breslow 2007). Additionally, *in vitro* studies have reported that SAHA directly inhibits the canine tumor cell line growth, including a study that demonstrated SAHA to have an antiproliferative effect on a hemangiosarcoma (Cohen et al. 2004, Kisseberth et al. 2008, Eto et al. 2019). However, little is known about how HDACi affects the canine immune system. Moreover, it is not clear whether HDACi influences the expression of NKG2DL in canines. Therefore, SAHA was used as an HDACi in this study to investigate its effect on PBMC cytotoxicity. Subsequently, the effects of SAHA on the mRNA expression of NKG2DL and the cytotoxicity of IL-2 activated PBMC in canine tumor cell lines were examined.

## Materials and Methods

### Cell lines and reagents

This study comprised three canine tumor cell lines as follows: a thyroid adenocarcinoma cell line (CTAC: purchased from ECACC, Salisbury, UK), a metastatic mammary gland tumor cell line (CIPm: gifted by Dr. Nakagawa, Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, University of Tokyo, Japan), and a melanoma cell line (MCM-N1: purchased from ECACC). Cultures of the cell lines were maintained in the RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100  $\mu$ g/ml streptomycin (GIBCO, NY, USA) at 37°C and 5% CO<sub>2</sub>. SAHA was purchased from Cayman Chemical (Michigan, USA), dissolved in DMSO, and used in this study.

### Real time-PCR

The mRNA expression levels were quantified using real-time PCR. The total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). The reverse transcription reaction was performed at 42°C for 50 min using the SuperScript II reverse transcriptase and oligo dT primer (Invitrogen, CA, USA).

Table 1. PCR primers used in this study.

Genes		Sequence (5'→3')	Product size	Source
NKG2DL	Forward	GTGGACAGTGCTTCAACCTG	248bp	014112758.2XM
	Reverse	TGGCACAGTAGAGAACAGG		
NKG2D	Forward	GTTATTGTGGTCCGTGTCCTAA	222bp	014108273.2XM
	Reverse	ACTGCCAGGATCCATTGTGG		
NKp46	Forward	CCAGCAGAGCCCCAAAACAAC	234bp	001284448.1NM
	Reverse	AGAC GGCAA-GAGAAGGTCAC		
NKp44	Forward	CCAAAGGAAGGGCTGGTGTA	209bp	035697676.1XM
	Reverse	CAGAGTTGCTGGAAGCATGG		
NK <sub>p</sub> 30	Forward	CTTCTTGCCGTGTTCCCTCAA	236bp	014118379.2XM
	Reverse	CCAGAACCTCCACTCTGCACA		
GAPDH	Forward	GATGGGCGTGAACCATGAG	13 lbp	001003142.2NM
	Reverse	TCATGAGGCCCTCCACGAT		
β-actin	Forward	GATATCGCTGCGCTTGTGG	272bp	001195845.2NM_
	Reverse	AGCTCGTTGTAGAAGGTGTGG		

The primers used for PCR amplification are listed in Table. 1. In this study, GAPDH and β-actin were selected as positive control genes. Real-time PCR was performed using the Step One™ PLUS Real-Time PCR System and POWER SYBR Green PCR Master Mix (Thermo Fisher Scientific, CA, USA) following the manufacturer's instructions. To confirm the specificity of the PCR, a single peak was investigated during the melt curve analysis. All samples were tested in duplicate. The mRNA expression level of the target gene was calculated as a relative value compared to the positive control gene using the ΔCt method.

### Peripheral blood mononuclear cell collection and culture

Whole-blood samples were procured from healthy beagles and were immediately heparinized. The beagles were kept in the Laboratory Animal Facility of the Kitasato University School of Veterinary Medicine. All the experiments were approved by the Laboratory Animal Ethics Committee of the Kitasato University. The dogs were not exposed to any drug for 6 months before the experiments. PBMC were separated from heparinized blood using the method described below. Blood diluted two-fold with physiological saline was gently layered on SEPARATE-L (specific gravity: 1.077, Muto Pure Chemicals, Tokyo, Japan) and centrifuged at 400 × g for 30 min. The PBMC layer was then collected, suspended in PBS, and washed by centrifugation twice at 300 × g for 10 min each. The PBMC obtained were suspended in Alys505S media (Cell Science & Technology Institute, Sendai, Japan) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and then incubated for 2 h at 37°C and 5% CO<sub>2</sub>. To generate activated PBMC, only non-

-adherent PBMC were collected, and cultured with the Alys505S media containing 500 U/ml IL-2, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin for 48 h at 37°C and 5% CO<sub>2</sub>. Fresh medium was added 48 h after the start of culture. In some experiments, fresh medium and 1 μM SAHA were added 48 h after the start of culture, followed by 48 h of culture. As vehicle controls, DMSO was added. The cultured PBMC were harvested, washed, and used as effector cells.

### Cytotoxicity assay

The cytotoxic activity of the cultured PBMC against tumor cell lines was evaluated using a standard lactate dehydrogenase (LDH) release assay, where LDH is released upon cell lysis. The tumor cell lines cultured in the presence or absence of 1 μM SAHA for 48 h were used as target cells and were seeded at a density of 4×10<sup>3</sup> cells/well in a 96-well U-bottom microplate. Mixtures of effector cells (E) and target cells (T) at E/T ratios of 40, 20, and 10 in conditions of 5% CO<sub>2</sub> at 37°C for 6 h were assayed. The cytotoxicity of effector cells to target cells was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. After culturing, the culture medium was harvested, and the absorbance at 490 nm of the wells was measured to analyze the LDH production. The experiment was performed in triplicates. The cytotoxic activities were calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{(\text{experimental release} - \text{effector spontaneous release} - \text{target spontaneous release})}{(\text{target maximum release} - \text{target spontaneous release})}$$

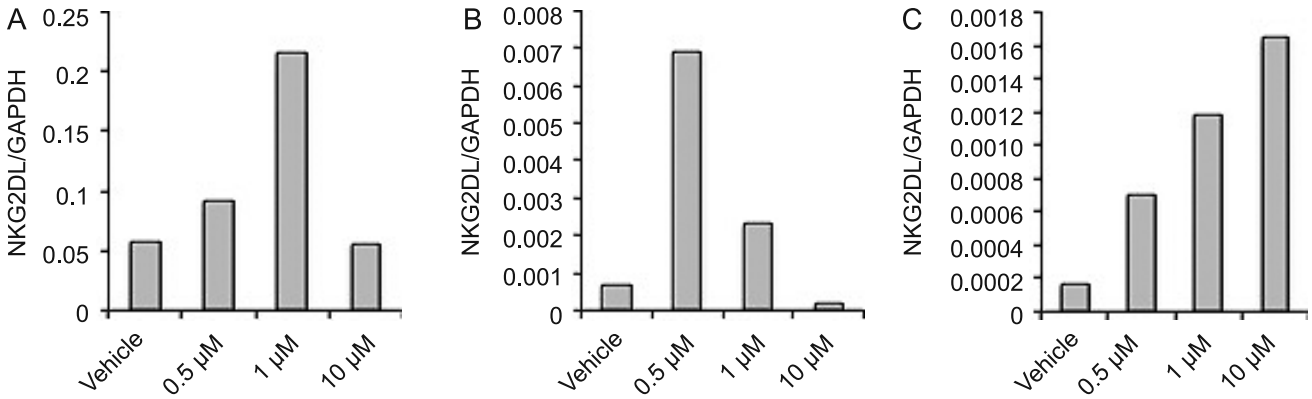


Fig. 1. The NKG2DL mRNA expressions in cell lines.

The expressions in CTAC (A), CIPm (B), and MCM-N1 (C) treated with a vehicle (DMSO) or 0.5–10  $\mu\text{M}$  SAHA for 48 hours have been shown. Representative data have been presented as the mean of relative value to GAPDH mRNA. Similar results were obtained in two or three independent experiments.

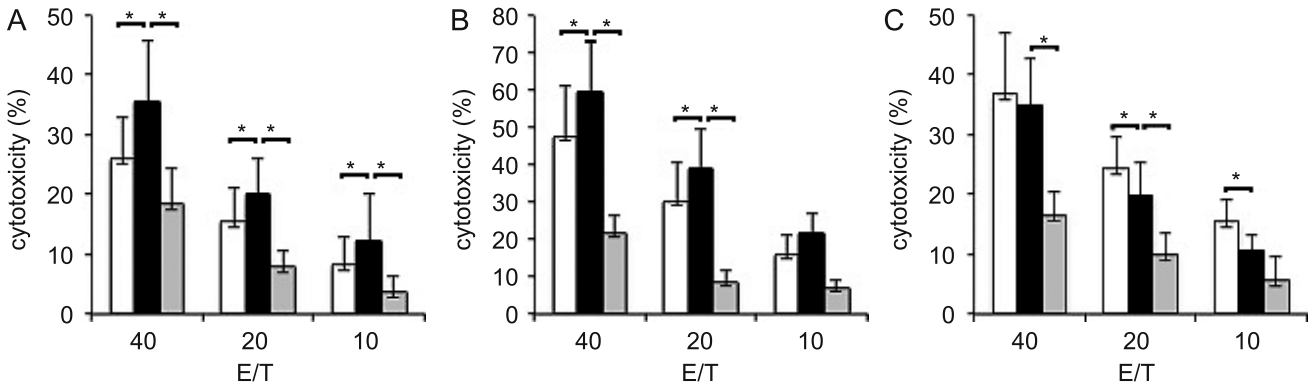


Fig. 2. Influence of SAHA on the cytotoxicity of activated PBMC.

White, black, and gray bars indicate the cytotoxicity of activated PBMC towards the vehicle-treated cell lines, that of cytotoxicity of activated PBMC toward 1  $\mu\text{M}$  SAHA-treated cell lines, and that of 1  $\mu\text{M}$  SAHA-treated activated PBMC toward 1  $\mu\text{M}$  SAHA-treated cell lines, respectively. A: CTAC (n=4), B: CIPm (n=6) and C: MCM-N1 (n=4). Each experiment was performed in triplicates, and the data are represented as mean  $\pm$  standard deviation. The asterisk shows the statistical significance obtained by the Student's *t*-test (\*  $p < 0.05$ ).

## Results

### Effect of SAHA on the NKG2DL mRNA expression in tumor cell lines

NKG2DL mRNA expression in the tumor cell lines cultured in the presence of 0–10  $\mu\text{M}$  SAHA for 48 h was quantified using real-time PCR. The data showed that SAHA upregulated NKG2DL mRNA in all the cell lines used in the present study (Fig. 1). However, NKG2DL mRNA expression in CTAC and CIPm was downregulated by the addition of 10  $\mu\text{M}$  and 1  $\mu\text{M}$  SAHA, respectively. On the other hand, the expression of NKG2D mRNA in MCM-N1 increased in a concentration-dependent manner.

### Influence of SAHA on activated PBMC cytotoxicity

The PBMC cytotoxicities, activated with IL-2 against the tumor cell lines cultured in the presence or

absence of SAHA were measured. All the tumor cell lines were killed in an activated PBMC number-dependent manner. The cytotoxicity towards CTAC and CIPm cultured in the presence of SAHA was significantly higher than that toward the cell lines cultured in the absence of SAHA (Fig. 2A, B). Surprisingly, the cytotoxicity towards MCM-N1 cultured in the presence of SAHA was reduced compared to the cytotoxicity towards MCM-N1 cultured in the absence of SAHA (Fig. 2C). Subsequently, a mixed culture of activated PBMC and tumor cell lines, both pretreated with 1  $\mu\text{l}$  SAHA for 48 hours were performed to examine the effect of SAHA on the activated PBMC. The results indicated that the treatment of the activated PBMC with SAHA significantly reduced the cytotoxicity toward all the cell lines compared to that of the non-treated activated PBMC (Fig. 3A-C).

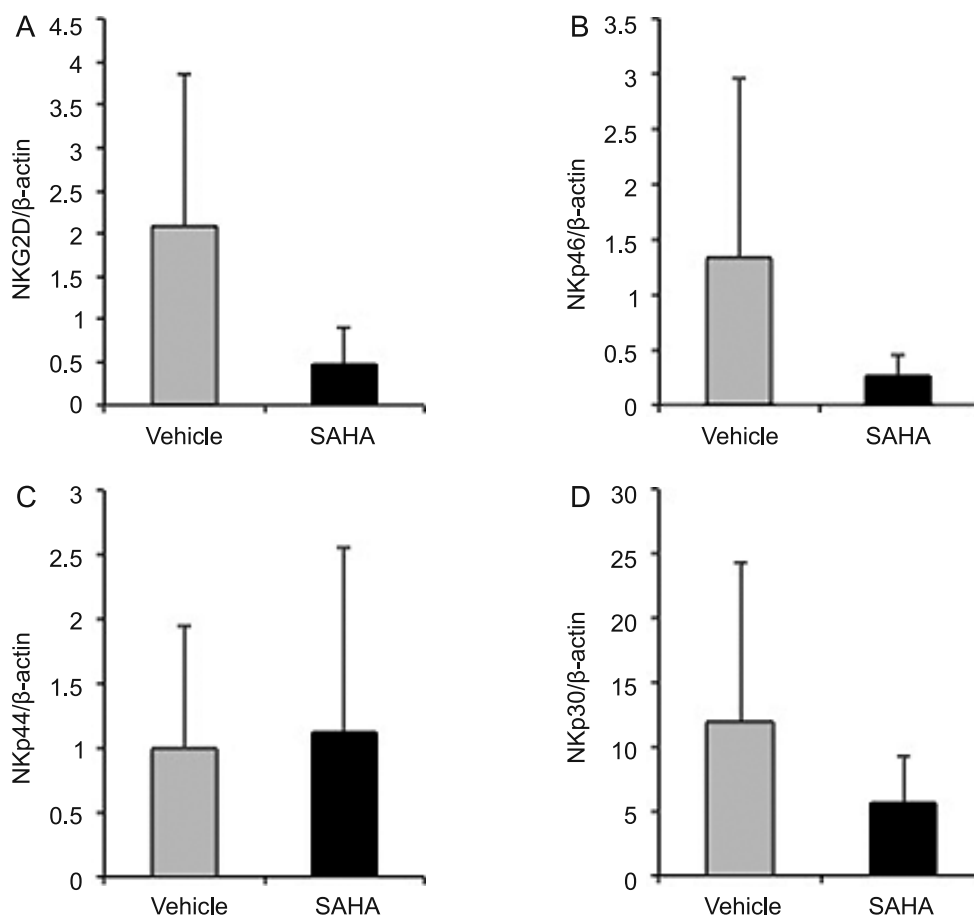


Fig. 3. The mRNA expression of NK trigger receptors in the SAHA-treated activated PBMC.

The mRNA expression levels of NKG2D (A), NKp46 (B), NKp44 (C), and NKp30 (D) in the activated PBMC treated with vehicle or 1  $\mu$ M SAHA is shown. Each value has been represented as the mean  $\pm$  standard deviation of the relative value to  $\beta$ -actin (n=4).

#### mRNA expression of NK trigger receptors in the activated PBMC cultured with SAHA

The mRNA expressions of NK trigger receptors, including NKG2D, NKp46, NKp44, and NKp30 in the activated PBMC that were cultured with IL-2 for 48 h followed by additional culture with 1  $\mu$ M SAHA for 48 h were quantified using real-time PCR. Although SAHA treatment reduced the mRNA expression of NKG2D, NKp46, and NKp30, a significant reduction could not be detected (Fig. 3).

#### Discussion

The present study revealed that the NKG2DL mRNA expression in canine tumor cells increased with SAHA treatment. Furthermore, it was demonstrated that the canine tumor cells pretreated with SAHA were sensitized to the cytotoxicity of activated PBMC, whereas activated PBMC directly pretreated with SAHA significantly decreased their cytotoxicity.

Many types of NKG2DL have been identified in humans and mice (Bauer et al. 1999, Cosman et al. 2001,

Cerwenka et al. 2001, Diefenbach et al. 2001, Li et al. 2001). Although some types of NKG2DL are expected to be present in canines, there are currently no identified canine NKG2D ligands. The NKG2DL gene amplified by real-time PCR was derived from the predicted sequence registered in the GenBank. However, NKG2DL expression is likely to be regulated at the post-transcriptional level, with no correlation between the mRNA and protein levels of NKG2D (Cosman et al. 2001, Radosavljevic et al. 2002, Raulet 2003). Therefore, it should be noted that the NKG2DL gene detected in this study may be a pseudogene that may not be translated into a functional protein.

The results revealed that SAHA could sensitize canine tumor cells to activated PBMC cytotoxicity; consistent with the reports in humans using IL-2 activated PBMC (Skov et al. 2005) or NK cells (Armeanu et al. 2005) as immune effector cells. This sensitization has been explained as being the result of enhanced expression of NKG2DL in the tumor cells by HDACi treatment, thereby partially enhancing the recognition of tumor cells by the NK cells. It is noteworthy that the NK cells functioned as the major cytotoxic effector

cells in the activated PBMC in this study. Because differentiation of alloreactive CD8<sup>+</sup> T cells into cytotoxic T lymphocytes (CTLs) takes several days (Schmudde et al. 2008), the 6-hour co-culture of activated PBMC and tumor cell lines in this study would not be sufficient to induce CTLs. Therefore, it is likely that the cell surface expression of NKG2DL detected by real-time PCR might be upregulated by SAHA, contributing to the partial increase of NK cell target recognition via the NKG2D-NKG2DL interaction.

As an exception, the cytotoxicity of activated PBMC toward MCM-N1 pretreated with SAHA was attenuated compared to that observed in the MCM-N1 not treated with SAHA. Although the attenuation could not be explained in this study, this result could be explained by a mechanism to escape tumor immunity. It has been reported that tumor cells, by releasing NKG2DL molecules from the cell surface as soluble proteins, interfere with the target recognition of CD8 T cells, NK cells, and  $\gamma\delta$  T cells, and induce downregulation of NKG2D expression in these immune effector cells (Maeda et al. 2000, Groh et al. 2002, Salih et al. 2003). Therefore, rather than increasing the cell surface expression of NKG2DL molecules, SAHA possibly promotes MCM-N1 to release the soluble NKG2D ligand molecule resulting in the attenuation of the NKG2D-NKG2DL interaction. This may result in the resistance of activated PBMC to the cytotoxicity.

However, the cytotoxicity mentioned above did not consider the effect of SAHA on the immune effector cells themselves. While HDACi has been shown to barely affect the normal cells, they reportedly attenuate the cytotoxicity of PBMC and NK cells (Ogbomo et al. 2007, Schmudde et al. 2010, Rossi et al. 2012, Shi et al. 2016). Very low concentrations of SAHA that do not result in tumor toxicity were reported to reduce PBMC cytotoxicity (Schmudde et al. 2010). The addition of SAHA during PBMC activation significantly attenuated the cytotoxicity of the activated PBMC. Therefore, even if SAHA sensitizes tumor cells to the cytotoxicity of PBMC, this effect may possibly have been counteracted by the inhibitory action of SAHA on the activated PBMC.

HDACi induces apoptosis in NK cells; however, the main cause of the reduction in PBMC cytotoxicity by HDACi has been described to be HDACi-induced down-regulation of NKG2D and NCRs in NK cells (Ogbomo et al. 2007, Schmudde et al. 2010, Rossi et al. 2012, Shi et al. 2016). Assuming that a similar down-regulation is involved in the present study, the mRNA expression of NKG2D and NCRs in activated PBMC treated with SAHA were measured. Although mRNA expression of these receptors in activated PBMC decreased with SAHA treatment, no significant

down-regulation of these receptor expressions was detected. Owing to the lack of tools for detecting these receptors at the protein level in canines, the effect on the protein expression remains unclear.

Therefore, it is necessary to consider not only the direct tumor-killing effect of SAHA but also a regimen that avoids the functional attenuation of immune effector cells by SAHA. It has been suggested that the attenuation of immune effector cell cytotoxicity may depend upon the time of HDACi addition in the activation culture. Some studies have reported that activation of NK cells in the presence of SAHA for 3-4 days significantly impaired NK cytotoxicity (Ogbomo et al. 2007, Schmudde et al. 2010). On the other hand, it has been reported that PBMC activation with IL-2 for 1-2 days followed by an additional culture with SAHA and IL-2, partially reduced the inhibitory effect of SAHA on PBMC proliferation and cytotoxicity (Schmudde et al. 2010). In this study, PBMC were cultured under conditions similar to those reported by Schmudde et al. (2010); however, the cytotoxicity was still significantly reduced. Therefore, it may be possible that this attenuation of cytotoxicity may depend on insufficient pre-activation of PBMC before the addition of SAHA, or an excess concentration of SAHA.

In conclusion, this study showed the effect of SAHA on NKG2DL expression and sensitization of tumor cells to PBMC cytotoxicity in canines. However, a negative effect of SAHA on the attenuation of PBMC cytotoxicity was also shown. Therefore, if this inhibitory action could be avoided, SAHA could be a beneficial approach for antitumor therapy in veterinary medicine.

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