JLB

Article

Histone deacetylase inhibitors impair NK cell viability and effector functions through inhibition of activation and receptor expression

Lucas E. Rossi, Damián E. Avila,*,† Raúl G. Spallanzani,* Andrea Ziblat,* Mercedes B. Fuertes,* Lara Lapyckyj,‡ Diego O. Croci,* Gabriel A. Rabinovich,*,§ Carolina I. Domaica,* and Norberto W. Zwirner*,‖,1*

Laboratorios de *Inmunopatología and ‡Estudios de la Interacción Celular en Reproducción y Cáncer, Instituto de Biología y Medicina Experimental (IBYME), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina; †Cátedra de Inmunología, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina; and Departamentos de §Química Biológica, Facultad de Ciencias Exactas y Naturales, and ‖Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

RECEIVED JULY 6, 2011; REVISED OCTOBER 20, 2011; ACCEPTED OCTOBER 21, 2011. DOI: 10.1189/jlb.0711339

ABSTRACT

HDACi are being used as a novel, therapeutic approach for leukemias and other hematological malignancies. However, their effect on immune cells remains ill-defined, as HDACi may impair immune surveillance. In this work, we demonstrate that TSA, VPA, and NaB inhibited IFN-γ production by CD56^{dim} and CD56^{bright} NK cells and NK cell-mediated cytotoxicity against K562 target cells. HDACi promoted minor NK cell apoptosis but inhibited nuclear mobilization of NF-κB p50, which was accompanied by a robust down-regulation of NKG2D and NKp46 on resting NK cells and of NKG2D, NKp44, NKp46, and CD25 on cytokine-activated NK cells. Decreased CD25 expression promoted a weakened IFN-γ secretion upon restimulation of NK cells with IL-2, whereas reduced expression of NKG2D and NKp46 was accompanied by an impaired NKG2D- and NKp46-dependent cytotoxicity. Moreover, NK cells from normal mice treated in vivo with TSA displayed a diminished expression of NK1.1, NKG2D, and NKp46 and secreted reduced amounts of IFN-γ upon ex vivo stimulation with cytokines. Thus, our preclinical results indicate that HDACi exert deleterious effects on NK cell function, which may weaken immune surveillance and facilitate relapse of the malignant disease in HDACi-treated patients. *J. Leukoc. Biol.* 91: 321–331; 2012.

Abbreviations: γ_c=γ chain, APC=allophycocyanin, AU=arbitrary unit(s), Cy=cyano dye, DNAM-1=DNAX accessory molecule-1, FC=flow cytometry, HDAC=histone deacetylase, HDACi=HDAC inhibitor, IC=isotype-matched negative control mAb, MFI=mean fluorescence intensity, MICA/MICB=MHC class I chain-related protein A/B, NaB=sodium butyrate, NCR=natural cytotoxicity receptor, NKG2DL=ligand for the NKR NKG2D, NKR=NK cell receptors, SAHA=suberoylanilide hydroxamic acid, SFI=specific fluorescence index, TSA=trichostatin A, ULBP=UL-16-binding protein, VPA=sodium valproate

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Cancer arises as a result of an accumulation of genetic mutations and epigenetic anomalies regulated by HDACs, which control chromatin condensation and gene expression [1]. Abnormal expression of HDACs has been reported in tumors, whereas knockdown of HDACs inhibits tumor growth [2, 3]. Thus, HDACi for treatment of cancer have been developed [4, 5]. SAHA (Vorinostat) and TSA are broad inhibitors of all HDACs, whereas VPA and NaB preferentially target class I and class IIa HDACs [4, 6]. They exhibit in vitro antiproliferative effects, inducing cell-cycle arrest, apoptosis, and autophagy, and are promising agents for the treatment of leukemias and lymphomas [4–7]. SAHA has been approved by the U.S. Food and Drug Administration for the treatment of cutaneous T cell lymphoma [6], and others are in advanced phases of clinical trials [5].

NK cells play a major role in tumor rejection, triggering cytotoxicity and IFN-γ secretion upon sensing target cells through activating receptors, such as NKG2D, DNAM-1, 2B4, and the NCRs NKp46, NKp44, and NKp30 [8, 9], or upon sensing proinflammatory stimuli [10]. In vitro, HDACi sensitize tumor cells to NCR-, DNAM-1-, and NKG2D-dependent cytotoxicity by promoting up-regulation of specific ligands on tumor cells, but in most cases, HDACi-treated tumor cells were confronted with untreated NK cells [11–14]. However, during treatment of patients, immune cells also become exposed to HDACi. In addition, clinical success of VPA is not superior to other pharmacologic strategies [15], which could be a result of effects of HDACi on immune cells. Different HDACi have been shown to reduce serum levels of proinflammatory cyto-

1. Correspondence: Laboratory of Immunopathology, IBYME-CONICET, Vuelta de Obligado 2490, C1428ADN Ciudad de Buenos Aires, Argentina. E-mail: nzwirner@ibyme.conicet.gov.ar

kines and acute graft-versus-host disease in mice [16], to expand regulatory T cells [17], and to inhibit macrophage, DC, and T cell activation and functions [18–20]. Thus, they may weaken immune surveillance and facilitate tumor relapse. Therefore, the aim of this work was to investigate the effect of TSA, VPA, and NaB on NK cell viability, functional response, and phenotype. Our results show that these HDACi severely impair NK cell activation, receptor expression, and effector functions, suggesting that they may deteriorate NK cell immune surveillance, a fact that may promote relapse in treated patients.

MATERIALS AND METHODS

Cell lines

HCT116 (colorectal carcinoma), T47D (mammary gland ductal carcinoma), and K562 (chronic myelogenous leukemia) human cell lines and P815 (mouse mastocytoma) were from American Type Culture Collection (Manassas, VA, USA). M8 (melanoma) was provided by Dr. Edgardo Carosella (Hôpital St. Louis, Paris, France).

Antibodies and reagents

Human and mouse rIL-2, rIL-12, and rIL-15 were from PeproTech (Rocky Hill, NJ, USA); human and mouse rIL-18 were from MBL International (Woburn, MA, USA). The following mAb against human molecules were used: anti-MICA/B mAb D7 [21]; anti-ULBP-1–3 mAb (clones 170,818, 165,903, and 166,510, respectively, R&D Systems, Minneapolis, MN, USA); anti-NKG2D mAb (clone 1D11, eBioscience, San Diego, CA, USA); anti-NKp30 (clone AZ20), anti-NKp44 (clone Z231), and anti-NKp46 (clone BAB281, kindly provided by Dr. Alessandro Moretta, Laboratory of Molecular Immunology, Department of Experimental Medicine, University of Genoa, Italy); anti-NF-κB p50 (clone E-10, Santa Cruz Biotechnology, Santa Cruz, CA, USA); IC (eBioscience); FITC-, PE-, and Spectral Red-labeled anti-CD3 mAb (clone UCHT-1, Southern Biotech, Birmingham, AL, USA); PE/Cy7-labeled anti-CD3 mAb (clone UCHT-1, eBioscience); PE/Cy5-labeled anti-CD56 (clone N901, Beckman Coulter, Brea, CA, USA); PE-labeled anti-CD11b (clone ICRF44, eBioscience); FITC-labeled anti-CD107a (clone 1D4B, Becton Dickinson, San Diego, CA, USA); PE-labeled anti-IFN-γ (clone 4S.B3, eBioscience); Alexa Fluor 488-labeled anti-CD226 (DNAM-1, clone DX11, BioLegend, San Diego, CA, USA); PE-labeled anti-CD244 (2B4, clone C1.7, BioLegend); PE-labeled anti-CD25 (clone BC96, eBioscience); FITC-labeled anti-CD69 (clone FN50, Becton Dickinson); and PE-labeled anti-pSTAT4 (clone py693, Becton Dickinson). The following mAb against mouse molecules were used: APC-labeled anti-NKG2D (clone CX5, eBioscience); PE-labeled anti-NK1.1 (clone PK136, eBioscience); PE-labeled anti-CD49b (clone DX5, eBioscience); Alexa Fluor 647-labeled anti-NKp46 (clone 29A1.4, eBioscience); and FITC-labeled anti-CD3 (clone 17A2, BioLegend). For indirect immunofluorescence staining and FC, PE-labeled goat anti-mouse IgG (Dako, Dakocytomation, Glostrup, Denmark) was used. For confocal microscopy, FITC-labeled goat anti-mouse (BD PharMingen, San Diego, CA, USA) was used. TSA, VPA, and NaB were from Enzo Life Sciences (Farmingdale, NY, USA). HDACi were dissolved in DMSO.

Mice and in vivo treatment with TSA

Normal C57BL/6 mice (6–8 weeks) were obtained from the animal facility of the School of Veterinary, National University of La Plata (Argentina). For in vivo treatment, TSA was dissolved in DMSO and i.p.-injected daily with 2 mg/kg during 4 days. At Day 5, spleen NK cells were isolated by positive selection.

NK cells and CD4 T cells

Human NK cells and CD4 T cells were isolated from blood of healthy volunteers (provided by the Hemotherapy Unit, Hospital Carlos G. Durand, Buenos Aires, Argentina) using the specific RosetteSep kits (Stemcell Technologies, Vancouver, BC, Canada) and Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ, USA) centrifugation. Purity of isolated cells was always above 90%, as assessed by FC ($CD3^-CD56^+$ for NK cells; $CD3^+CD4^+$ for CD4 T cells). Mouse NK cells were obtained from spleens using CD49b (DX5) MicroBeads (positive selection kit from Miltenyi Biotec, Auburn, CA, USA). Purity of isolated cells was always above 80%, as assessed by FC ($CD3^-NK1.1^+$ cells). NK cells were cultured in the absence or in the presence of different HDACi for 24 h, as indicated in each figure, in the absence or in the presence of 20 ng/ml of IL-12, 20 ng/ml IL-15, and 20 ng/ml IL-18 in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% inactivated FBS (NatoCor, Córdoba, Argentina), sodium pyruvate, glutamine, and gentamicin (Sigma-Aldrich). In some experiments, NK cells were cultured for 24 h with 10 ng/ml IL-15 and TSA, then the HDACi was removed, and NK cells were washed and cultured further with 10 ng/ml IL-15 as a survival factor in the absence of TSA for an additional period of 24–72 h. In other experiments, NK cells were cultured for 24 h with 10 ng/ml IL-12, 10 ng/ml IL-15, and 10 ng/ml IL-18; TSA was removed; and NK cells were washed and further cultured with 10 ng/ml IL-12, 10 ng/ml IL-15, and 10 ng/ml IL-18 in complete medium in the absence of TSA for 24–72 h. Also, NK cells were cultured in the absence or in the presence of 1 μM TSA, 2 mM VPA, or 2 mM NaB for 24 h; washed; and stimulated with K562 cells for 24 h at a ratio of 1:3 (NK cell:K562 cells). In addition, NK cells were activated with IL-12, IL-15, and IL-18 for 4 days, TSA was added to the cultures, and cells were cultured for another 24 h in the presence of the cytokines. In other experiments, TSA-treated, activated NK cells were cultured for 24 h with 5 ng/ml IL-2 to assess the functional role of CD25. Treated and untreated NK cells were used for analysis of apoptosis, IFN-γ production, degranulation, and cell surface expression of specific receptors and activation markers. Recombinant human cytokines were used for experiments with human NK cells, whereas recombinant mouse cytokines were used for experiments with mouse NK cells. CD4 T cells were cultured in the absence or in the presence of TSA for 24 h. Then, apoptosis was analyzed as explained below. In all experiments, NK or CD4 T cells cultured in the absence of HDACi were cultured in the presence of an equivalent volume of DMSO—the vehicle used to dissolve the drug. Studies have been approved by the institutional review committee.

FC

Expression of NKG2DLs on cell lines and of NKRs on NK cells was analyzed by FC using specific mAb and PE-labeled anti-mouse IgG. For mouse NKRs, fluorochrome-labeled mAb were used. NK cells were characterized as $CD3^-CD56^+$ (human cells) or $CD3^-NK1.1^+$ (mouse cells) using fluorochrome-labeled mAb. For phosphorylated STAT4 detection, cells were fixed and permeabilized with Cytofix/Cytoperm (Becton Dickinson) and incubated with the anti-pSTAT4 mAb or an IC mAb following the instructions provided by the manufacturer. Cells were analyzed in a FACSAria flow cytometer (Becton Dickinson). SFI was calculated as the ratio of the MFI of the specific mAb and the MFI of the isotype control mAb.

Apoptosis

Apoptosis was assessed using Annexin V conjugated to FITC (Becton Dickinson) or APC (eBioscience) or the apoptosis detection kit (Becton Dickinson), following the manufacturer's instructions.

ELISA for IFN-γ

Secretion of IFN-γ by human NK cells was analyzed by ELISA as described [22]. Detection of IFN-γ secretion by mouse NK cells was performed using the OptEIA kit from Becton Dickinson.

NK cell degranulation and IFN-γ production

NK cells were cultured overnight at 37°C in the absence or in the presence of IL-12, IL-15, and IL-18 or K562 target cells, with or without HDACi. During the last 4 h, the anti-CD107a or IC mAb were added together with the Golgi-Plug reagent (Becton Dickinson). Cells were harvested and stained with anti-CD3 and anti-CD56 mAb, permeabilized with Cytofix/Cytoperm (Becton Dickinson), and stained with anti-IFN-γ mAb to determine the percentage of IFN-γ^+CD107a$^-$, IFN-γ^+CD107a$^+$, or IFN-γ^-CD107a$^+$ cells within the CD3$^-$CD56$^+$ population.

Cytotoxicity

NK cells were cultured for 18 h with 10 ng/ml IL-15 in the absence or in the presence of different HDACi, washed extensively, and cocultured overnight with K562 cells at a 1:1 ratio. Cells were then labeled with Annexin V-APC and analyzed by FC. Apoptosis of K562 cells (Annexin V-positive cells) was assessed. K562 cells were distinguished from NK cells by their different forward- versus side-scatter parameters (Supplemental Fig. 1). For the redirected killing assay, P815 cells were coated with anti-NKG2D, anti-NKp46, or IC mAb, washed, and incubated overnight with NK cells, previously untreated or treated for 24 h with HDACi. Thereafter, cells were stained with anti-CD56-PE/Cy5 to gate out NK cells and with Annexin V-FITC to assess apoptosis in P815 cells by FC in the tumor cell population, gated by forward- versus side-scatter parameters (Supplemental Fig. 1).

Indirect immunofluorescence and confocal microscopy analysis

NK cells were seeded onto polylysine-coated glass coverslips, stimulated for 2 h with 20 ng/ml IL-12, 20 ng/ml IL-15, and 20 ng/ml IL-18 in the absence or in the presence of 1 μM TSA, fixed with 2% of PFA, permeabilized with 0.1% saponin for 10 min, and stained with anti-p50 mAb and FITC-labeled goat anti-rabbit IgG. Slides were mounted with Dabco, and cells were observed in a digital Eclipse E800 Nikon C1 laser confocal microscope with Nikon Plan Apo 60X/1.40 Oil objective.

RNA extraction real-time PCR

Total RNA was isolated with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was synthesized with oligo-dT and SuperScript III RT (Invitrogen Life Technologies). Primers for the amplification of NKG2DL and cytokine receptors, as well GAPDH (housekeeping gene), are listed in **Table 1**. Quantitative assessment of each amplified mRNA was performed by real-time PCR with the 7500 Real-Time PCR unit and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All samples were run in duplicates, and negative controls (without template) were included in all cases. To confirm specificity of the signal observed in real-time protocols, melting curves were constructed and analyzed in each run. Expression levels of each template were calculated using standard curves built with serial dilutions of cDNA. Data were normalized against GAPDH expression and expressed as AU.

TABLE 1. Primers Used for the Real-Time PCR for MICA, ULBP-2, and Cytokine Receptors

Gene product	Primer	Sequence
MICA	Forward	AACGGAAAGGACCTCAGGAT
	Reverse	AGCTCCTGGTGCTGTTGTCT
ULBP-2	Forward	GTGCAGGAGCACCACTCGCC
	Reverse	AGGACTCTCCTCAGATGCCAGGGA
IL-12Rβ1	Forward	CGGACACCCAGCAGCCCATG
	Reverse	GGGGTTTTCAGGGGGAACGCA
IL-15Rβ	Forward	CCTGAGGGTGCTGTGCCGTG
	Reverse	GGGGCCATCAGGCGAAGGTT
IL-2Rγ (γ_c)	Forward	GGAACGGACGATGCCCCGAA
	Reverse	AGACACACCACTCCAGGCCGAA
IL-18R	Forward	GCTGTGGAGATTTTGCCCAGGGTG
	Reverse	ACAACAGCTCCTCCAGGCACTACAT
GAPDH	Forward	TGCACCACCAACTGCTTAGC
	Reverse	GGCATGGACTGTGGTCATGAG

Statistical analysis

A paired Student's *t* test was used to compare the percentage of IFN-γ^+CD107a$^-$, IFN-γ^+CD107a$^+$, or IFN-γ^-CD107a$^+$ cells; receptor expression in human NK cells treated with a single dose of HDACi; and IFN-γ secretion by mouse NK cells stimulated ex vivo with cytokines in the presence or in the absence of TSA. An unpaired Student's *t* test with Welch's correction was used to compare viability, receptor expression, and IFN-γ secretion by mouse NK cells from TSA- versus DMSO-treated animals. One-way ANOVA with Dunnett's post hoc test was used for comparison of IFN-γ secretion and receptor expression in the presence of different concentrations of HDACi. One-way ANOVA with Bonferroni's correction was used for comparison of the percentage of IFN-γ-producing NK cells, the percentage of apoptotic NK cells in the absence or in the presence of different HDACi, and the percentage of cytotoxicity of HDACi-exposed NK cells toward K562 cells.

RESULTS

HDACi inhibit NK cell-mediated cytotoxicity and IFN-γ production

First, we investigated whether TSA regulates cell surface expression of NKG2DLs on human tumor cell lines. As shown in **Fig. 1A**, TSA induced up-regulation of MICA/B and ULBP-2 in HCT116, M8, and T47D cells, whereas expression of ULBP-1 was up-regulated slightly only in T47D cells. Expression of ULBP-3 was also only up-regulated slightly in HCT116 and K562 cells (not shown). TSA-induced, up-regulated expression of MICA and ULBP-2 was confirmed by real-time PCR (Fig. 1B for MICA and Fig. 1C for ULBP-2), suggesting that the effect of TSA is exerted at a transcriptional level. These results confirm that TSA up-regulates NKG2DLs on tumor cells, as was described previously by others.

Next, we investigated whether TSA and other HDACi with a narrower specificity (VPA and NaB) affect the functional response of human NK cells. As shown in **Fig. 2A**, all three HDACi strongly inhibited IFN-γ secretion of NK cells stimulated with IL-12, IL-15, and IL-18. CD56^{dim} and CD56^{bright} NK cells were affected by the HDACi (Fig. 2B). By FC, we observed that stimulation of NK cells with IL-12, IL-15, and IL-18 induced the appearance of an 8.8 ± 2.7% of IFN-γ^+CD107a$^-$ cells, a 9.5 ± 4.8% of IFN-γ^+CD107a$^+$ cells, and a 2.5 ± 1.0% of IFN-γ^-CD107a$^+$ cells. However, treatment with TSA reduced such percentages to 0.2 ± 0.1%, 0.2 ± 0.1%, and 1.3 ± 1.0%, respectively (Fig. 2C). TSA also affected the response of NK cells against tumor cells, as a coculture with K562 cells induced the appearance of a 3.3 ± 2.0% of IFN-γ^+CD107a$^-$ cells, a 3.7 ± 3.0% of IFN-γ^+CD107a$^+$ cells, and a 12.0 ± 5.1% of IFN-γ^-CD107a$^+$ cells, but treatment with TSA diminished such percentages to 1.6 ± 0.9%, 0.6 ± 0.4%, and 6.2 ± 4.3%, respectively (Fig. 2C). The decrease in the percentage of IFN-γ^+CD107a$^-$ cells, IFN-γ^+CD107a$^+$, and IFN-γ^-CD107a$^+$ cells induced by TSA in NK cells stimulated with cytokines and the decrease in the percentage of IFN-γ^-CD107a$^+$ cells in-

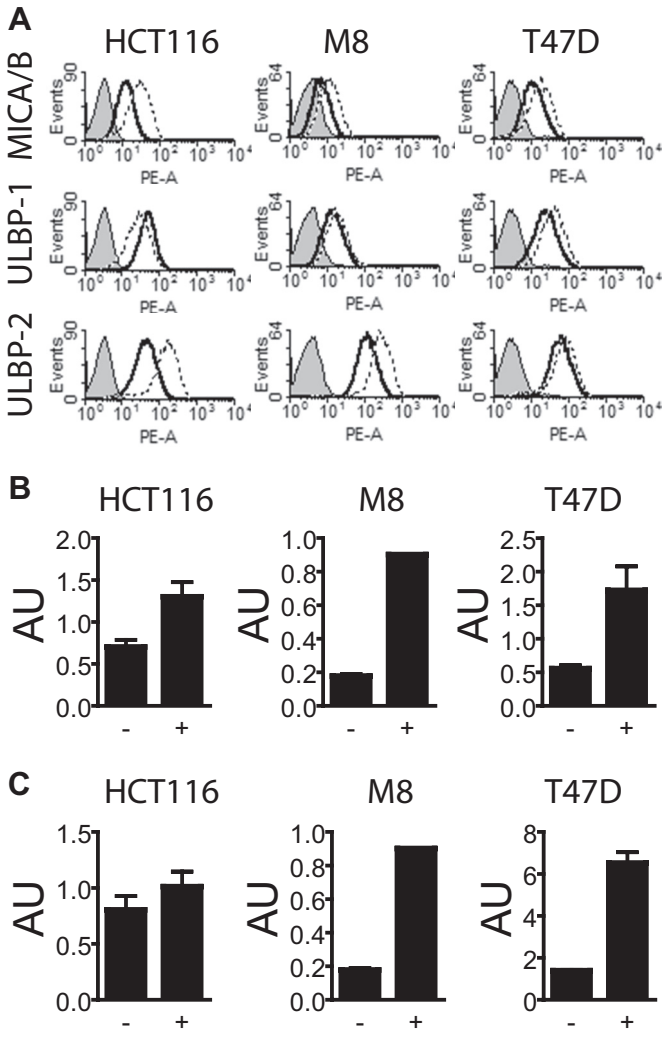

Figure 1. TSA up-regulates cell surface expression of NKG2DLs on tumor cell lines. (A) Cell lines indicated at the top of each column of histograms were cultured in the absence (black lines) or in the presence (dashed lines) of TSA 1 μM for 24 h, and surface expression of human NKG2DLs (indicated at the left of each row of histograms) was investigated by FC. Gray histograms, IC mAb. Results are representative of three independent experiments. PE-A, PE-Area. (B and C) Cell lines indicated at the top of each graph were cultured in the absence (–) or in the presence (+) of TSA 1 μM for 24 h, and expression of MICA (B) or ULBP-2 (C) was investigated by real-time PCR. Results correspond to the mean ± SEM from three independent experiments.

duced by TSA in NK cells stimulated with K562 cells were statistically significant. Also, in cocultures of NK cells with K562 cells, we observed significantly reduced percentages of Annexin V^+ K562 cells after exposure to HDACi-treated NK cells versus untreated NK cells (Fig. 2D) and significantly less IFN-γ secreted by NK cells treated with these HDACi (Fig. 2E), suggesting that TSA, VPA, and NaB impair NK cell-mediated cytotoxicity and IFN-γ secretion upon recognition of tumor target cells.

As impaired NK cell function could be a result of HDACi-induced NK cell death, we assessed the percentage of apoptotic and secondary necrotic NK cells upon exposure to TSA, VPA, or NaB (**Fig. 3A**). However, we observed a slight increase in Annexin V^+ NK cells induced by the three HDACi. Remarkably, CD4 T cells were much less susceptible to TSA-induced apoptosis than resting or cytokine-stimulated NK cells (Fig. 3B), which exhibited a slight increase in early (Annexin V^+IP^- cells) and late (Annexin V^+IP^+ cells) apoptotic NK cells. These results suggest that NK cells exhibit a differential susceptibility to HDACi-induced apoptosis. Also, we did not observe induction of secondary necrosis of NK cells in our experimental conditions, as we did not detect Annexin V^-IP^+ cells.

Stimulation of NK cells with cytokines triggers intracellular pathways that converge in NF-κB activation. When NK cells were stimulated with IL-12, IL-15, and IL-18 in the presence of TSA, we observed an almost complete inhibition of nuclear mobilization of NF-κB p50 (**Fig. 4A**).

Overall, these results suggest that while promoting up-regulated cell surface expression of NKG2DLs on tumor cells, HDACi severely impair NK cell-mediated IFN-γ secretion and cytotoxicity but induce a minor proapoptotic effect, as >60% of the NK cells remained viable after exposure to TSA for 24 h, whereas >80% of the NK cells remained viable after exposure to VPA or NaB.

HDACi down-regulate cell surface expression of NKRs

As we observed an intense, immunosuppressive effect of HDACi on NK cell effector functions, accompanied by a minor, proapoptotic effect, we investigated additional mechanisms triggered by these HDACi that account for such a striking, functional impairment. Thus, we examined whether these compounds compromised expression of cytokine receptors (Fig. 4B) and the major activating receptors (**Fig. 5**). Exposure of NK cells to TSA significantly inhibited the expression of IL-12Rβ1, IL-15Rβ, IL-2Rγ (γ_c), and IL-18R chains, as assessed by real-time PCR (Fig. 4B). In addition, treatment of resting NK cells with TSA, VPA, or NaB for 24 h significantly reduced cell surface NKG2D and NKp46 in a dose-dependent manner, without affecting the expression of NKp30 (Fig. 5A and B for TSA, Fig. 5C and D for VPA, and Fig. 5E and F for NaB). The reduction in cell surface expression of these receptors was 76 ± 3.9% for NKG2D and 73.2 ± 1.4% for NKp46 with 1 μM TSA, 73.6 ± 3.6% for NKG2D and 45.8 ± 7.3% for NKp46 with 10 mM VPA, and 67.4 ± 7.1% for NKG2D and 47.0 ± 7.7% for NKp46 with 10 mM NaB. These three HDACi also significantly inhibited in a dose-dependent manner the cell surface expression of NKG2D and NKp46 in NK cells simultaneously stimulated with IL-12, IL-15, and IL-18 (Fig. 5A and B for TSA, Fig. 5C and D for VPA, and Fig. 5E and F for NaB). In this case, the reduction in cell surface expression of the receptors was 86.3 ± 2.6% for NKG2D and 51.8 ± 5.7% for NKp46 with 1 μM TSA, 70.0 ± 7.2% for NKG2D and 31.5 ± 8.8% for NKp46 with 10 mM VPA, and 58.4 ± 11.2% for NKG2D and 58.0 ± 3.3% for NKp46 with 10 mM NaB. As well, DNAM-1 and 2B4 expression was also down-regulated significantly by TSA but not by VPA or NaB (not shown).

To address whether the effects of TSA were transient or long-lasting, NK cells were treated for 24 h with TSA or

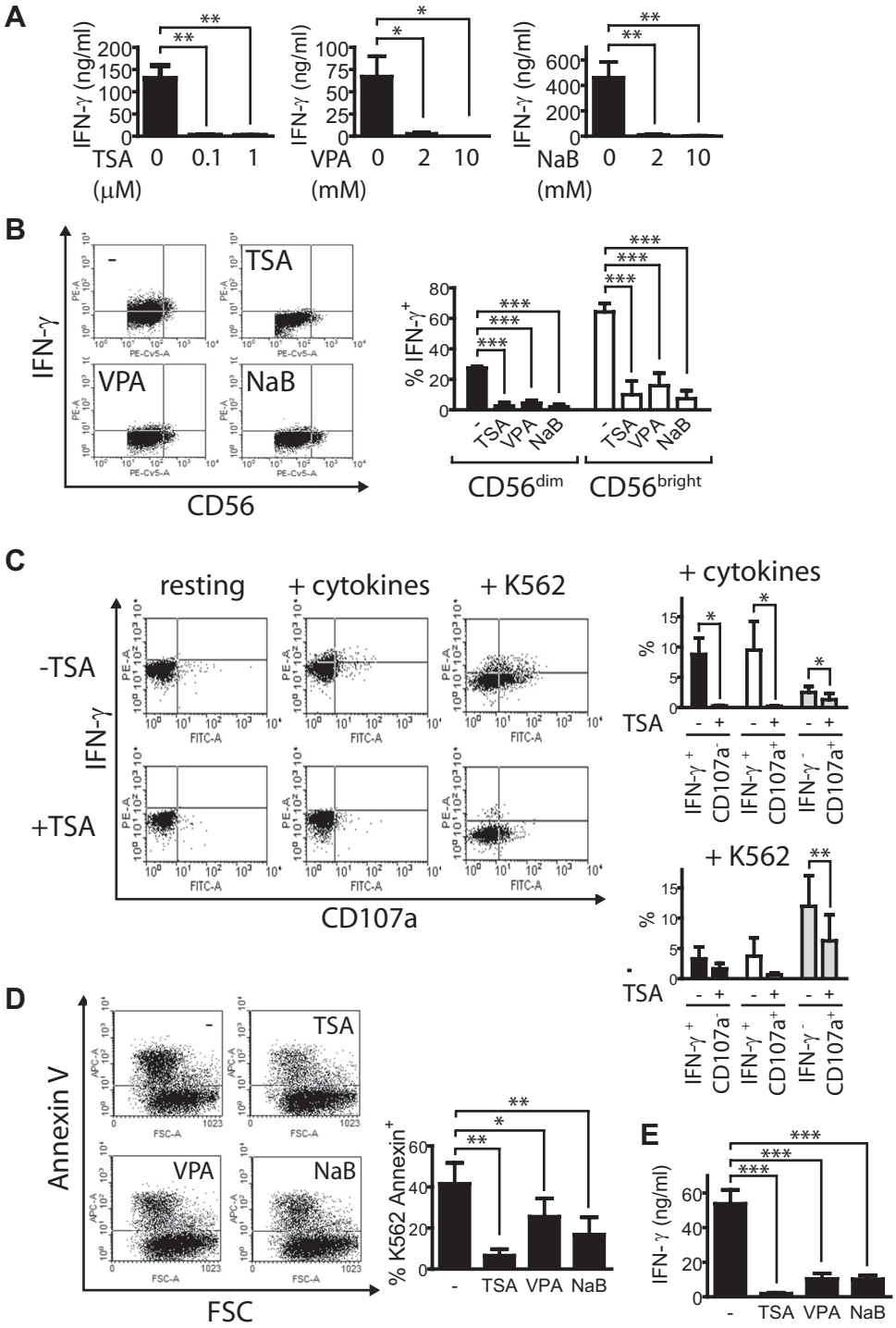

Figure 2. TSA, VPA, and NaB impair human NK cell effector functions. (A) Human NK cells were cultured with IL-12 + IL-15 + IL-18 for 24 h in the absence or in the presence of different doses of TSA (left graph), VPA (middle graph), or NaB (right graph). Then, IFN-γ production was assessed by ELISA. (B) NK cells were stimulated with IL-12 + IL-15 + IL-18 for 24 h in the absence (–) or in the presence of 1 μM TSA, 2 mM VPA, or 2 mM NaB. Then, IFN-γ production was assessed by FC in $CD56^{dim}$ and $CD56^{bright}$ cells. Representative dot plots are shown on the left, and mean ± SEM obtained with NK cells from four independent donors is shown on the right. (C) Human NK cells were cultured without stimulus ("resting") or with IL-12 + IL-15 + IL-18 ("+cytokines") or K562 cells ("+K562") for 24 h in the absence (–TSA) or in the presence (+TSA) of 1 μM TSA. Then, intracellular IFN-γ and cell surface CD107a were assessed on $CD56^+$ cells by FC. Results shown on the left are representative of four independent experiments with NK cells from different donors. Results on the right correspond to the mean ± SEM from such experiments. Black bars, IFN-γ^+CD107a$^-$ NK cells; white bars, IFN-γ^+CD107a$^+$ NK cells; gray bars, IFN-γ^-CD107a$^+$ NK cells. (D) NK cells were cultured overnight without or with HDACi as in B and used for assessment of cytotoxicity against K562 cells. Dot plots on the left are representative experiments. Graphs on the right show the mean ± SEM obtained with NK cells from four independent donors. FSC, Forward scatter. (E) NK cells were cultured for 24 h in the absence (–) or in the presence of 1 μM TSA, 2 mM VPA, or 2 mM NaB, washed, and cocultured for 24 h with K562 cells at a ratio of 1:3 (NK cell:K562 cells). Then, IFN-γ secretion was assessed in cell culture supernatants by ELISA. Graphs show the mean ± SEM obtained with NK cells from four independent donors. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

treated for 24 h with TSA and cultured for 24–72 h in the absence of this HDACi. Then, expression of NKG2D and NKp46 and IFN-γ secretion were analyzed (**Fig. 6**). Although TSA strongly inhibited cell surface expression of NKG2D and NKp46 upon 24 h, NK cells started to re-express both receptors when the drug was removed, and the expression of both receptors was essentially identical to expression in untreated cells upon 72 h or 48 h of culture in the absence of TSA, respectively (Fig. 6A). Such behavior was mirrored by a recovery in NK cell-mediated IFN-γ secretion in response to cytokines upon withdrawal of TSA (Fig. 6B). These results indicate that inhibitory effects of HDACi on receptor expression and IFN-γ secretion by NK cells are enduring but reversible.

Next, we investigated whether NK cells stimulated for longer periods with cytokines also are affected by HDACi. Therefore, NK cells were cultured for 4 days with IL-12, IL-15, and IL-18 and then exposed to TSA for 24 h. We observed that activated NK cells exhibited statistically significant, reduced expression

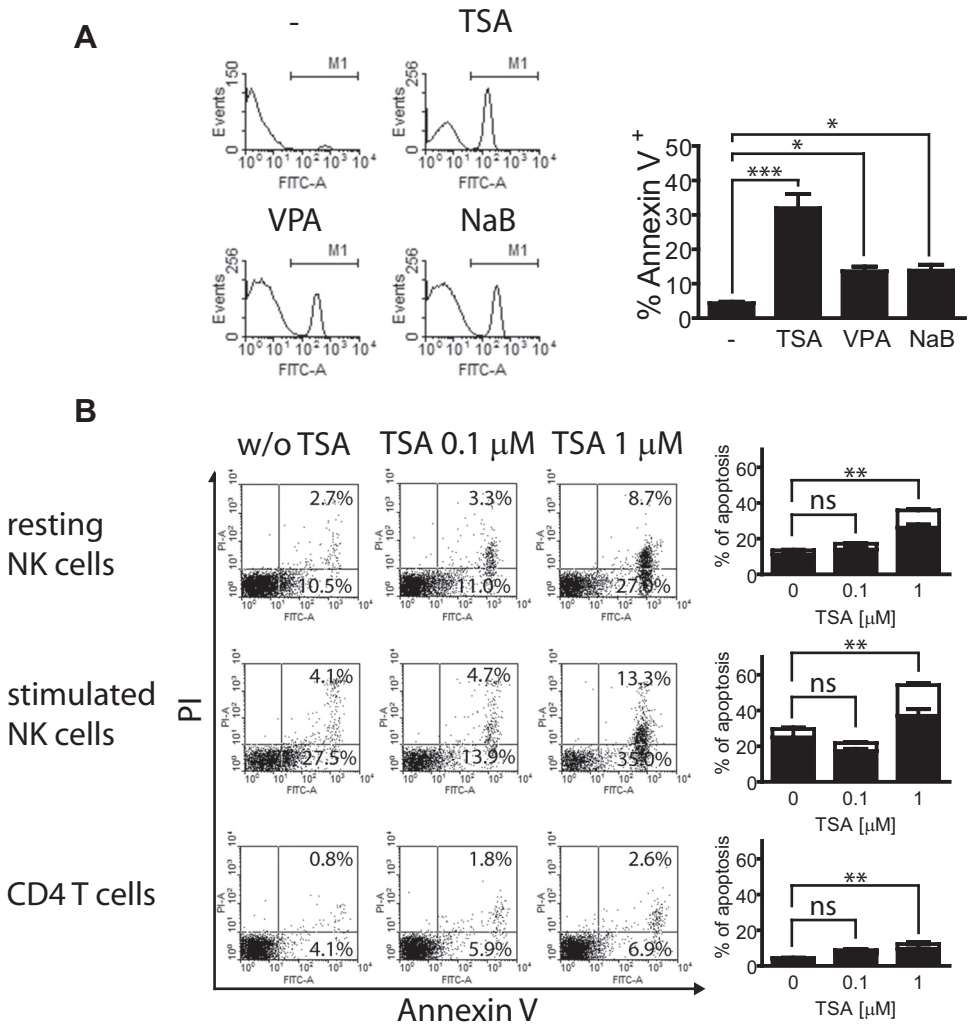

Figure 3. HDACi induce a low degree of apoptosis in human NK cells. (A) NK cells were cultured for 24 h without or with 1 μM TSA, 2 mM VPA, or 2 mM NaB, and apoptosis was assessed by Annexin V staining in the CD56$^+$ population. Representative histograms are shown on the left. Mean ± SEM obtained with NK cells from four independent donors are shown on the right. (B) Resting NK cells, NK-stimulated for 24 h with IL-12, IL-15, and IL-18, or CD4 T cells were cultured for 24 h in the absence (w/o TSA) or in the presence of 0.1 μM or 1 μM TSA, and early and late apoptosis and secondary necrosis were assessed by Annexin V and PI staining. Representative dot plots are shown on the left (percentage of Annexin V$^+$IP$^-$ cells and Annexin V$^+$IP$^+$ cells are indicated in the respective quadrants). Mean ± SEM obtained with NK cells from four independent donors are shown on the right. The percentage of early (Annexin V$^+$PI$^-$ cells, black bars) and late (Annexin V$^+$PI$^+$ cells, white bars) apoptotic cells was depicted, and the statistical comparison was performed using the total (early+late) percentage of apoptotic cells. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

of NKG2D, NKp44, and NKp46, with reductions in cell surface expression of these three receptors of 77.4 ± 5.2% for NKG2D, 48.0 ± 15.0% for NKp44, and 56.6 ± 7.2% for NKp46 (**Fig. 7A**). Also, TSA selectively down-regulated expression of CD25 without affecting expression of CD69 (Fig. 7B), suggesting that this HDACi affects the preservation of the activated phenotype in NK cells. TSA-induced CD25 down-regulation was accompanied by an impaired responsiveness to IL-2, as TSA-treated, activated NK cells secreted less IFN-γ than activated NK cells not exposed to the drug (Fig. 7C). Thus, TSA-driven down-regulation of CD25 is physiologically relevant.

To assess the physiological relevance of HDACi-induced down-regulation of NKG2D and NKp46, we performed redirected killing assays with P815 cells coated with anti-NKG2D or anti-NKp46 mAb (**Fig. 8**). We observed that HDACi-treated NK cells triggered significantly lower NKG2D- and NKp46-mediated cytotoxicity of P815 cells than untreated NK cells, indicating that HDACi-mediated receptor down-regulation is physiologically relevant.

Overall, our results indicate that HDACi selectively impair the expression of NK cell-activating receptors on resting NK cells, during the activation of NK cells with cytokines and on activated NK cells. Also, these compounds impair expression of CD25 on cytokine-stimulated NK cells and their ability to further respond to IL-2.

The inhibitory effects of TSA compromise NK cell activity in vivo

To validate our results in vivo, first, we analyzed the effect of TSA, VPA, and NaB on IFN-γ secretion by mouse NK cells stimulated in vitro with IL-12 + IL-15 + IL-18 (**Fig. 9A**). We observed that these HDACi strongly inhibited IFN-γ secretion by mouse NK cells. Next, normal C57BL/6 mice were injected daily during 4 days with TSA, and 24 h later, NK cells were isolated, and IFN-γ secretion was analyzed upon ex vivo stimulation with IL-12, IL-15, and IL-18. We observed that NK cells from mice treated with TSA produced less IFN-γ upon ex vivo stimulation with IL-12, IL-15, and IL-18 than NK cells from mice treated with DMSO (the vehicle used to dissolve the drug; Fig. 9B) and that these NK cells cultured ex vivo for 4 days with IL-12 + IL-15 + IL-18 in the absence of TSA recovered their ability to secrete IFN-γ (Fig. 9C), thus substantiating the reversible nature of this effect. Consistent with in vitro findings, NK cells isolated from spleens of mice treated with

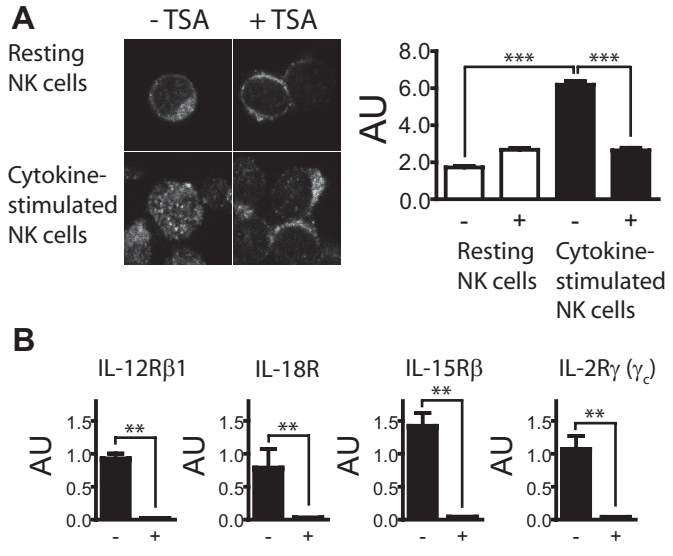

Figure 4. TSA inhibits nuclear mobilization of NF-κB p50 and cytokine receptor expression in NK cells. (A) Human NK cells were cultured alone ("Resting NK cells") or with IL-12 + IL-15 + IL-18 ("Cytokine-stimulated NK cells") in the absence ("–TSA") or in the presence ("+TSA") of 1 μM TSA for 2 h. Then, NK cells were processed for NF-κB p50 detection and analyzed by confocal microscopy. Results presented correspond to NK cells from one experiment performed with cells from three independent donors. At least 100 cells/condition were analyzed. (B) Expression of IL-12Rβ1, IL-15Rβ, IL-2Rγ (γ_c), and IL-18R was analyzed by real-time PCR in NK cells that were cultured in the absence (–) or in the presence (+) of 1 μM TSA. Graphs show mean intensity expressed in AU ± SEM obtained with NK cells from three different donors. For statistic analysis and as a result of heterogeneity in the SDs, a logarithmic transformation of data was applied. $^{**}P < 0.01$; $^{***}P < 0.001$.

TSA exhibited a larger fraction of cells with apoptotic morphology (smaller size and higher granularity) than NK cells isolated from spleens of mice treated with DMSO (not shown). Also, morphologically viable NK cells from TSA-treated mice exhibited lower surface expression of the activating receptors NK1.1, NKG2D, and NKp46, as compared with NK cells from mice treated with DMSO (Fig. 9D). Compared with control mice, the reduction on cell surface expression of the activating receptors on NK cells was 35.0 ± 4.8% for NK1.1, 22.5 ± 5.9% for NKG2D, and 8.8 ± 2.9% for NKp46. Thus, suppressive effects of HDACi on NK cells also occur in vivo upon treatment with TSA and involve impaired secretion of IFN-γ, alteration of NK cell morphology, and down-regulation of major activating receptors involved in target cell recognition, validating the relevance of our in vitro findings with human NK cells.

DISCUSSION

Since the discovery that tumors exhibit altered HDAC activity [2, 3] and that interference with the expression or activity of HDACs inhibits tumor growth and survival, different HDACi have been developed for the treatment of cancer [4–7, 23]. Although it has been claimed that HDACi minimally affect

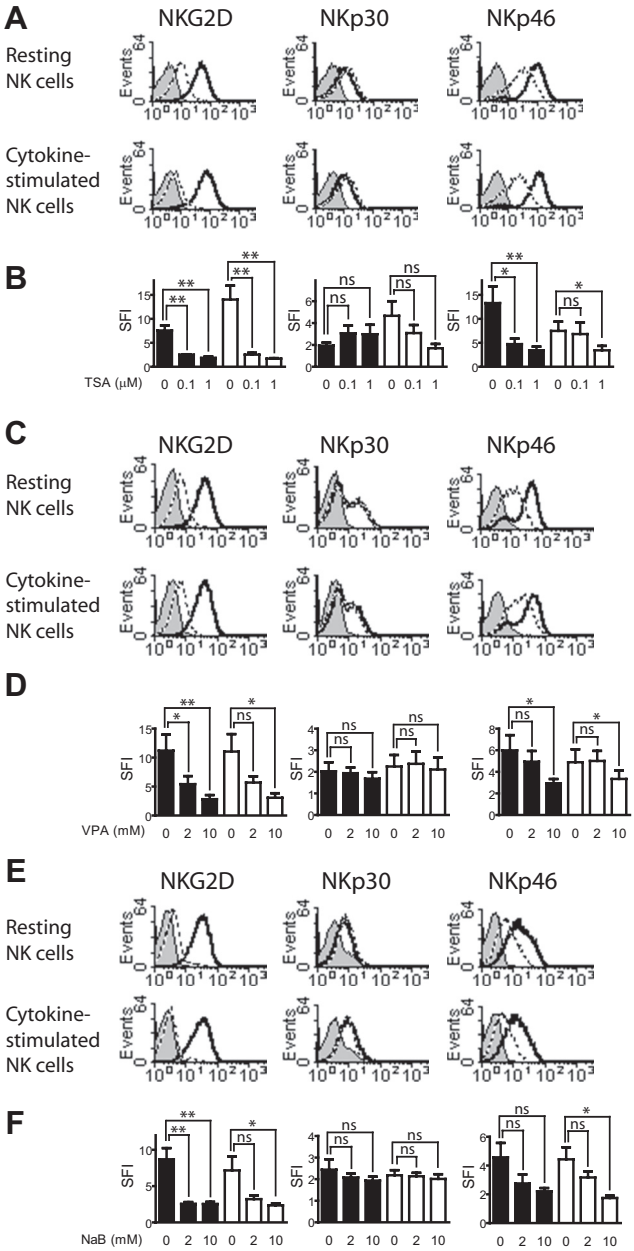

Figure 5. HDACi down-regulate activating receptors on resting and cytokine-stimulated NK cells. Human NK cells were cultured without stimulus (Resting NK cells) or were stimulated with IL-12 + IL-15 + IL-18 for 24 h (Cytokine-stimulated NK cells) in the absence or in the presence of 0.1 or 1 μM TSA (A and B), 2 or 10 mM VPA (C and D), or 2 or 10 mM NaB (E and F). Then, cell surface expression of NKG2D, NKp30, and NKp46 was assessed by FC on morphologically viable cells. Data shown in histograms depicted in A, C, and E are representative of four independent experiments performed with NK cells from different donors. Black lines, without HADCi; dashed lines, with 1 μM TSA (A), 10 mM VPA (C), or 10 mM NaB (E); gray histograms, IC mAb. Graphs shown in B, D, and F correspond to the mean SFI ± SEM obtained in such experiments for each receptor. Black bars, resting NK cells; white bars, cytokine-stimulated NK cells. $^{*}P < 0.05$; $^{**}P < 0.01$.

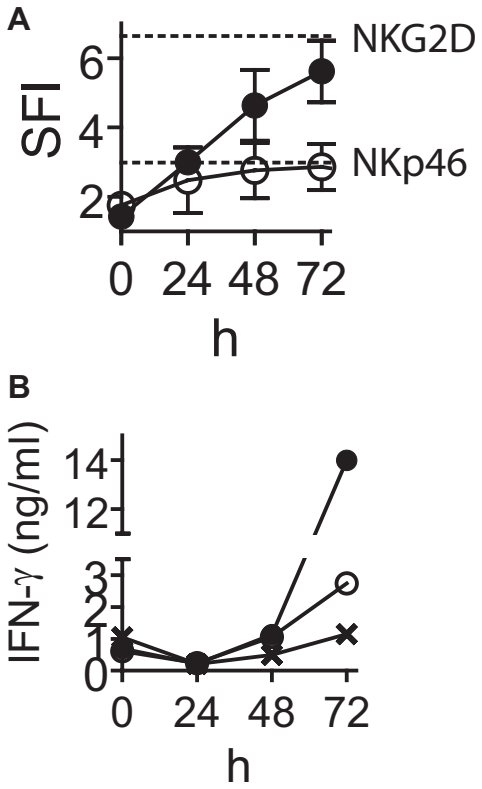

Figure 6. TSA-induced down-regulation of NKG2D and NKp46 and impairment of IFN-γ secretion are lasting but reversible. (A) Isolated human NK cells were cultured with 10 ng/ml IL-15 in the absence or in the presence of 1 μM TSA for 24 h. Then, NK cells were washed and cultured for 24–72 h (as indicated on the graph) in fresh medium with 10 ng/ml IL-15 as a survival factor in the absence of TSA, and cell surface expression of NKG2D and NKp46 was assessed by FC on morphologically viable cells. The mean ± SEM obtained with NK cells from three independent donors is depicted (● for NKG2D and ○ for NKp46). Solid lines, TSA-treated NK cells; dotted lines, expression of NKG2D and NKp46 on NK cells not exposed to the drug. (B) NK cells were cultured with 10 ng/ml IL-12, 10 ng/ml IL-15, and 10 ng/ml IL-18 in the presence of 1 μM TSA for 24 h. Then, NK cells were washed and cultured for 24–72 h (as indicated on the graph) in fresh medium with 10 ng/ml IL-12, 10 ng/ml IL-15, and 10 ng/ml IL-18 in the absence of TSA, and IFN-γ secretion was assessed by ELISA. Each line corresponds to a different donor.

normal cells, most experiments addressed their effects on viability and toxicity using fibroblasts [24, 25]. In vitro, HDACi increase expression of ligands for NKRs, such as NCRs, NKG2D, and DNAM-1, and tumor cell susceptibility to NK cell-mediated effector functions in tumor cell lines [11–15]. However, HDAC may also regulate epigenetic events in immune cells, including NK cells, and long-term effects of chronic administration of HDACi to human patients remain unknown.

Many clinical trials have been performed using TSA [4–7], a HDACi that is structurally similar to SAHA (Vorinostat). Both have identical, broad specificity for different HDACs. Conversely, VPA and NaB have a narrower specificity and also are being tested in clinical trials for the treatment of different tumors. Remarkably, VPA has been used for many years for the treatment of patients with epilepsy [26], which generated a debate about its safety and possible effects on cancer incidence. Strikingly, some data indicate that long-term users of VPA exhibit an increased incidence of tumors and leukemias [27–29] and polycystic ovary syndrome [30]. As NK cells are major players in immune surveillance against cancer, we undertook an analysis of the effects of different HDACi on NK cell effector functions. Although we confirmed that TSA induced the expression of NKG2DLs in human tumor cell lines, as has been shown by others [12, 14, 15, 31, 32], we demonstrated that the TSA, VPA, and NaB drastically inhibited IFN-γ production by human $CD56^{dim}$ and $CD56^{bright}$ NK cells stimulated with IL-12, IL-15, and IL-18 or by NK cells stimulated with K562 tumor cells. HDACi also inhibited NK cell degranulation, and NK cells exposed to TSA, VPA, or NaB exhibited a significantly reduced cytotoxicity against K562 cells. Therefore, HDACi seem to affect NK cell activation through cytokine receptors and through activating receptors involved in tumor cell recognition. Although resting and cytokine-stimulated NK cells were more susceptible to HDACi-induced apoptosis than CD4 T cells, the marked impairment of NK cell-mediated effector functions could not be explained by HDACi-induced NK cell apoptosis, as most NK cells remained viable after exposure to the drugs. Thus, we reasoned that HDACi exert additional suppressive effects that impair NK cell-mediated effector functions. A pivotal pathway triggered by IL-12, IL-15, and IL-18 is activation of NF-κB [33–35]. We observed that one of the earliest pathways affected by HDACi upon cytokine stimulation seems to be nuclear mobilization of p50, which is concordant with a rapid inhibitory effect of some HDACi on NF-κB activation reported in the literature [36–38].

NK cell-mediated cytotoxicity against tumor cells is triggered upon engagement of activating receptors [39, 40]. As TSA, VPA, and NaB strikingly impaired NK cell-mediated cytotoxicity, we investigated whether these HDACi affect cell surface expression of the major NK cell-activating receptors. In particular, we focused our attention on NKG2D, as it has been observed that NK cell-mediated killing of K562 cells is dependent on this receptor [41]. We observed that TSA, VPA, and NaB strongly down-regulated expression of NKG2D and NKp46 on resting NK cells and expression of NKG2D, NKp44, and NKp46 on NK cells stimulated with IL-12, IL-15, and IL-18. Expression of DNAM-1 and 2B4 was only affected by TSA. Down-regulation of NKG2D and NKp46 is functionally relevant, as HDACi-treated NK cells exhibited lower NKG2D- and NKp46-dependent cytotoxicity in redirected killing assays. In addition, suppressive effects on NKR expression and function were slowly reversed upon withdrawal of TSA. Activated NK cells exposed to TSA also expressed reduced amounts of NKG2D, NKp44, and NKp46 and exhibited lower amounts of CD25, which impaired responsiveness to IL-2, indicating that the effect is functionally relevant.

Previously, it was observed that HDACi can compromise NK cell-mediated cytotoxicity of NK cells stimulated for 3–4 days with IL-2 [42, 43], but the effect of HDACi on resting NK cells or during NK cell activation with IL-12, IL-15, and IL-18 and the effects on IFN-γ secretion were not investigated. These three cytokines

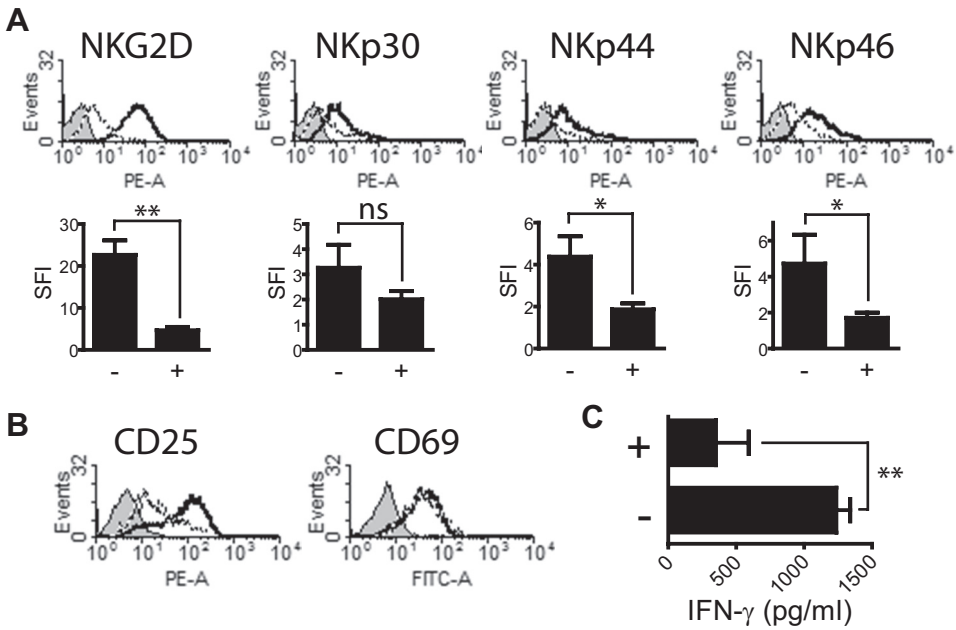

Figure 7. HDACi down-regulate activating receptors on activated NK cells. (A) NK cells were cultured for 4 days with IL-12 + IL-15 + IL-18, washed, and cultured for 24 h with these cytokines in the absence or in the presence of 1 μM TSA. Then, cell surface expression of NKG2D, NKp30, NKp44, and NKp46 was assessed by FC on morphologically viable cells. Data in histograms are representative of four independent experiments performed with NK cells from different donors. Black lines, Without TSA; dashed lines, with TSA 1 μM; gray histograms, IC mAb. Graphs show the mean SFI ± SEM obtained in such experiments for each receptor. (B) Expression of CD25 and CD69 on cytokine-activated NK cells (same pattern of histograms as in A). (C) NK cells were activated with IL-12, IL-15, and IL-18 for 4 days. Then, NK cells were cultured for 24 h in the absence (–) or in the presence (+) of 1 μM TSA with fresh cytokines, after which, NK cells were washed and cultured for 24 h with 5 ng/ml IL-2. Graphs correspond to the mean SFI ± SEM obtained with cells from four independent experiments performed with NK cells from different donors. $*P < 0.05$; $**P < 0.01$.

are critical during NK cell–DC cross-talk during the activation of the immune response, as they promote NK cell-mediated IFN-γ secretion, which in turn, promotes DC maturation and reciprocal activation of NK cells [39, 40, 44, 45]. We observed that HDACi almost completely abrogated IFN-γ secretion by NK cells but induced a low degree of apoptosis. As NK cell-derived IFN-γ contributes to DC maturation [44, 45], HDACi may also affect their cross-talk with NK cells. Also, although it has been observed that HDACi down-regulated NKp46 and NKp30 on NK cells activated with IL-2 [42], we observed down-regulation of NKG2D and NKp46 on resting NK cells and of NKG2D, NKp44, and NKp46 on NK cells stimulated with cytokines relevant for NK cell activation during the physiology of the immune response against infectious agents and tumors. This discrepancy might be a result of the different stimuli used in both works. Also, as activated NK cells exposed to TSA exhibited impaired responsiveness to IL-2 as a result of decreased expression of CD25, our results explain previous inhibitory effects of HDACi on NK cells stimulated with IL-2 [42].

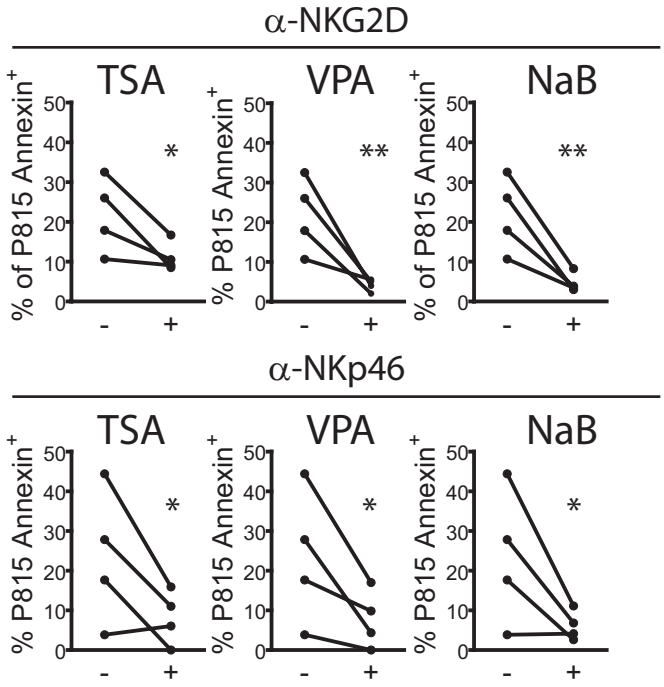

Figure 8. HDACi-treated NK cells exhibit impaired NKG2D- and NKp46-dependent cytotoxicity. NK cells were cultured without (–) or with (+) 1 μM TSA, 2 mM VPA, or 2 mM of NaB for 24 h. Then, NK cells were used in a redirected killing assay with P815 cells coated with anti-NKG2D or anti-NKp46 mAb, and the percentage of apoptotic P815 cells was assessed by Annexin V staining in the $CD56^-$ population by FC. Data corresponding to individual donors are shown. $*P < 0.05$; $**P < 0.01$.

Additionally, we validated our results in vivo, as NK cells from normal C57BL/6 mice treated with TSA for 4 days exhibited a reduced ability to secrete IFN-γ in response to cytokines and exhibited lower levels of NK1.1, NKG2D, and NKp46. Moreover, the in vivo effect of HDACi was reversed after 4 days in culture with IL-12, IL-15, and IL-18.

From a clinical perspective, our results suggest that long-term administration of HDACi may lead to a progressive decline in NK cell immune surveillance, creating a permissive scenario that may facilitate the relapse of the primary malignant disease, the generation of metastasis, and even the onset of a second malignancy. This idea is supported further by our findings that indicate that HDACi down-regulated expression of NKG2D and NKp46 on NK cells, as it was demonstrated by previous work of others that NKG2D and NKp46 knockout mice exhibit increased tumor initiation and progression [46–48]. Also, an in vivo mAb-mediated blockade of NKG2D promotes an increased tumor initiation in mice [49], and a correlation between natural cytotoxic activity of PBMCs and tumor incidence has been described in humans [50].

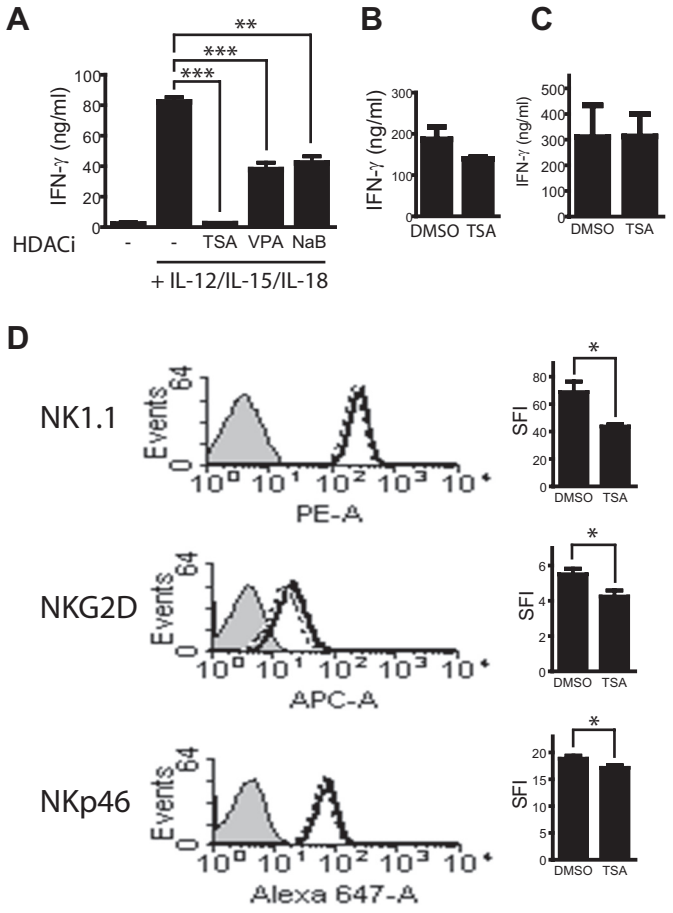

Figure 9. In vivo, TSA impairs IFN-γ secretion by mouse NK cells and down-regulates NKR expression. First, NK cells were isolated from normal C57BL/6 mice and cultured without or with 20 ng/ml IL-12, 20 ng/ml IL-15, and 20 ng/ml IL-18 in the absence or in the presence of 0.1 μM TSA, 1 mM VPA, or 1 mM NaB. Then, IFN-γ secretion was analyzed by ELISA (A). In addition, NK cells were isolated from normal C57BL/6 mice after 5 days of treatment with DMSO or with 2 mg/kg TSA. NK cells from both groups of animals were stimulated for 24 h (B) or 5 days (C) with IL-12, IL-15, and IL-18, and IFN-γ was assessed by ELISA. Also, expression of major NKRs (NK1.1, NKG2D, and NKp46) was assessed by FC on NK cells isolated from untreated (DMSO) or treated (TSA) animals (D). Representative histograms are shown on the left. Bar graphs show results of mean ± SEM obtained with four mice of each group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Therefore, our results may have important translational consequences, which may influence the clinical management of patients treated with HDACi, as assessment of NKR expression and effector functions may constitute surrogate biomarkers to detect an early decline in immune surveillance in HDACi-treated patients, which would facilitate the adoption of timely, corrective measures, such as interruption or modification of chemotherapy treatment. Analysis of these NK cell parameters also might be used during clinical trials with novel drugs to predict possible weakening of immune surveillance. In summary, we demonstrated in this work that NK cell-mediated IFN-γ secretion, degranulation, and cytotoxicity are dampened by HDACi as a result of induction of apoptosis of a minor fraction of NK cells, down-regulation of the major activating receptors, inhibition of proper activation, and impaired responsiveness to IL-2. Thus, our findings constitute a warning message about the safety of the use of HDACi in humans, and we suggest that the effect of these compounds on immune cells should be tested carefully before moving to a clinical setting, using appropriate preclinical models to closely monitor and identify possible deleterious effects on immune surveillance (in particular, on NK cells). Such an approach would allow the design and selection of improved compounds that minimally compromise immune surveillance, leading to better clinical success and reducing the incidence of relapse of a primary malignant disease, the establishment of metastasis, or the onset of second malignancies.

AUTHORSHIP

L.E.R. designed and performed the experimental work and analyzed data. D.E.A., R.G.S., A.Z., and C.I.D. performed experiments and revised the manuscript. L.L. assisted with the real-time PCR set-up, optimization, and analysis. D.O.C. assisted with the confocal microscopy experiments. G.A.R. and M.B.F. helped with the discussion of the results and revised the manuscript. N.W.Z. designed research and the conceptual framework, analyzed and interpreted data, supervised the work, and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the National Agency for Promotion of Science and Technology from Argentina (ANPCYT), the National Research Council of Argentina (CONICET), and the University of Buenos Aires (UBA) to N.W.Z. We thank Fundación Sales for additional support, Dr. Alessandro Moretta (University of Genoa, Italy) for providing the anti-NCR mAb, Dr. Edgardo Carosella (Hôpital St. Louis, Paris, France) for providing the M8 cell line, and Dr. Vargas and personnel from the Hemotherapy Unit (Hospital Carlos G. Durand, Buenos Aires, Argentina) for providing buffy coats. L.E.R., D.E.A., R.G.S., A.Z., C.I.D., L.L., and D.O.C. are fellows of CONICET. M.B.F., G.A.R., and N.W.Z. are members of the Researcher Career of CONICET.

DISCLOSURES

The authors declare that there are no conflicts of interests.

REFERENCES

1. Roth, S. Y., Denu, J. M., Allis, C. D. (2001) Histone acetyltransferases. *Annu. Rev. Biochem.* **70,** 81–120.
2. Zhu, P., Martin, E., Mengwasser, J., Schlag, P., Janssen, K. P., Gottlicher, M. (2004) Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. *Cancer Cell* **5,** 455–463.
3. Zhang, Z., Yamashita, H., Toyama, T., Sugiura, H., Omoto, Y., Ando, Y., Mita, K., Hamaguchi, M., Hayashi, S., Iwase, H. (2004) HDAC6 expression is correlated with better survival in breast cancer. *Clin. Cancer Res.* **10,** 6962–6968.
4. Bolden, J. E., Peart, M. J., Johnstone, R. W. (2006) Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug Discov.* **5,** 769–784.
5. Minucci, S., Pelicci, P. G. (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat. Rev. Cancer* **6,** 38–51.
6. Khan, O., La Thangue, N. B. (2008) Drug insight: histone deacetylase inhibitor-based therapies for cutaneous T-cell lymphomas. *Nat. Clin. Pract. Oncol.* **5,** 714–726.

7. Lane, A. A., Chabner, B. A. (2009) Histone deacetylase inhibitors in cancer therapy. *J. Clin. Oncol.* **27,** 5459–5468.
8. Moretta, L., Bottino, C., Pende, D., Castriconi, R., Mingari, M. C., Moretta, A. (2006) Surface NK receptors and their ligands on tumor cells. *Semin. Immunol.* **18,** 151–158.
9. Lanier, L. L. (2005) NK cell recognition. *Annu. Rev. Immunol.* **23,** 225–274.
10. Zwirner, N. W., Domaica, C. I. (2010) Cytokine regulation of natural killer cell effector functions. *Biofactors* **36,** 274–288.
11. Armeanu, S., Bitzer, M., Lauer, U. M., Venturelli, S., Pathil, A., Krusch, M., Kaiser, S., Jobst, J., Smirnow, I., Wagner, A., Steinle, A., Salih, H. R. (2005) Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. *Cancer Res.* **65,** 6321–6329.
12. Lopez-Soto, A., Folgueras, A. R., Seto, E., Gonzalez, S. (2009) HDAC3 represses the expression of NKG2D ligands ULBPs in epithelial tumor cells: potential implications for the immunosurveillance of cancer. *Oncogene* **28,** 2370–2382.
13. Schmudde, M., Braun, A., Pende, D., Sonnemann, J., Klier, U., Beck, J. F., Moretta, L., Broker, B. M. (2008) Histone deacetylase inhibitors sensitize tumor cells for cytotoxic effects of natural killer cells. *Cancer Lett.* **272,** 110–121.
14. Skov, S., Pedersen, M. T., Andresen, L., Straten, P. T., Woetmann, A., Odum, N. (2005) Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3-dependent expression of MHC class I-related chain A and B. *Cancer Res.* **65,** 11136–11145.
15. Poggi, A., Catellani, S., Garuti, A., Pierri, I., Gobbi, M., Zocchi, M. R. (2009) Effective in vivo induction of NKG2D ligands in acute myeloid leukemias by all-trans-retinoic acid or sodium valproate. *Leukemia* **23,** 641–648.
16. Reddy, P., Maeda, Y., Hotary, K., Liu, C., Reznikov, L. L., Dinarello, C. A., Ferrara, J. L. (2004) Histone deacetylase inhibitor suberoylanilide hydroxamic acid reduces acute graft-versus-host disease and preserves graft-versus-leukemia effect. *Proc. Natl. Acad. Sci. USA* **101,** 3921–3926.
17. Tao, R., de Zoeten, E. F., Ozkaynak, E., Chen, C., Wang, L., Porrett, P. M., Li, B., Turka, L. A., Olson, E. N., Greene, M. I., Wells, A. D., Hancock, W. W. (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat. Med.* **13,** 1299–1307.
18. Halili, M. A., Andrews, M. R., Labzin, L. I., Schroder, K., Matthias, G., Cao, C., Lovelace, E., Reid, R. C., Le, G. T., Hume, D. A., Irvine, K. M., Matthias, P., Fairlie, D. P., Sweet, M. J. (2010) Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS. *J. Leukoc. Biol.* **87,** 1103–1114.
19. Brogdon, J. L., Xu, Y., Szabo, S. J., An, S., Buxton, F., Cohen, D., Huang, Q. (2007) Histone deacetylase activities are required for innate immune cell control of Th1 but not Th2 effector cell function. *Blood* **109,** 1123–1130.
20. Nencioni, A., Beck, J., Werth, D., Grunebach, F., Patrone, F., Ballestrero, A., Brossart, P. (2007) Histone deacetylase inhibitors affect dendritic cell differentiation and immunogenicity. *Clin. Cancer Res.* **13,** 3933–3941.
21. Fuertes, M. B., Girart, M. V., Molinero, L. L., Domaica, C. I., Rossi, L. E., Barrio, M. M., Mordoh, J., Rabinovich, G. A., Zwirner, N. W. (2008) Intracellular retention of the NKG2D ligand MHC class I chain-related gene A in human melanomas confers immune privilege and prevents NK cell-mediated cytotoxicity. *J. Immunol.* **180,** 4606–4614.
22. Girart, M. V., Fuertes, M. B., Domaica, C. I., Rossi, L. E., Zwirner, N. W. (2007) Engagement of TLR3, TLR7, and NKG2D regulate IFN-γ secretion but not NKG2D-mediated cytotoxicity by human NK cells stimulated with suboptimal doses of IL-12. *J. Immunol.* **179,** 3472–3479.
23. Abujamra, A. L., Dos Santos, M. P., Roesler, R., Schwartsmann, G., Brunetto, A. L. (2010) Histone deacetylase inhibitors: a new perspective for the treatment of leukemia. *Leuk. Res.* **34,** 687–695.
24. Purrucker, J. C., Fricke, A., Ong, M. F., Rube, C., Rube, C. E., Mahlknecht, U. (2010) HDAC inhibition radiosensitizes human normal tissue cells and reduces DNA double-strand break repair capacity. *Oncol. Rep.* **23,** 263–269.
25. Ungerstedt, J. S., Sowa, Y., Xu, W. S., Shao, Y., Dokmanovic, M., Perez, G., Ngo, L., Holmgren, A., Jiang, X., Marks, P. A. (2005) Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. *Proc. Natl. Acad. Sci. USA* **102,** 673–678.
26. Johannessen Landmark, C. (2008) Antiepileptic drugs in non-epilepsy disorders: relations between mechanisms of action and clinical efficacy. *CNS Drugs* **22,** 27–47.
27. Hallas, J., Friis, S., Bjerrum, L., Støvring, H., Narverud, S. F., Heyerdahl, T., Grønbæk, K., Andersen, M. (2009) Cancer risk in long-term users of valproate: a population-based case-control study. *Cancer Epidemiol. Biomarkers Prev.* **18,** 1714–1719.
28. Coyle, T. E., Bair, A. K., Stein, C., Vajpayee, N., Mehdi, S., Wright, J. (2005) Acute leukemia associated with valproic acid treatment: a novel mechanism for leukemogenesis? *Am. J. Hematol.* **78,** 256–260.
29. Williams Jr., D. C., Massey, G. V., Russell, E. C., Riley, R. S., Ben-Ezra, J. (2008) Translocation-positive acute myeloid leukemia associated with valproic acid therapy. *Pediatr. Blood Cancer* **50,** 641–643.
30. Duncan, S. (2001) Polycystic ovarian syndrome in women with epilepsy: a review. *Epilepsia* **42,** 60–65.
31. Diermayr, S., Himmelreich, H., Durovic, B., Mathys-Schneeberger, A., Siegler, U., Langenkamp, U., Hofsteenge, J., Gratwohl, A., Tichelli, A., Paluszewska, M., Wiktor-Jedrzejczak, W., Kalberer, C. P., Wodnar-Filipowicz, A. (2008) NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. *Blood* **111,** 1428–1436.
32. Kato, N., Tanaka, J., Sugita, J., Toubai, T., Miura, Y., Ibata, M., Syono, Y., Ota, S., Kondo, T., Asaka, M., Imamura, M. (2007) Regulation of the expression of MHC class I-related chain A, B (MICA, MICB) via chromatin remodeling and its impact on the susceptibility of leukemic cells to the cytotoxicity of NKG2D-expressing cells. *Leukemia* **21,** 2103–2108.
33. Grohmann, U., Belladonna, M. L., Bianchi, R., Orabona, C., Ayroldi, E., Fioretti, M. C., Puccetti, P. (1998) IL-12 acts directly on DC to promote nuclear localization of NF-κB and primes DC for IL-12 production. *Immunity* **9,** 315–323.
34. McDonald, P. P., Russo, M. P., Ferrini, S., Cassatella, M. A. (1998) Interleukin-15 (IL-15) induces NF-κB activation and IL-8 production in human neutrophils. *Blood* **92,** 4828–4835.
35. Fortin, C. F., Ear, T., McDonald, P. P. (2009) Autocrine role of endogenous interleukin-18 on inflammatory cytokine generation by human neutrophils. *FASEB J.* **23,** 194–203.
36. Place, R. F., Noonan, E. J., Giardina, C. (2005) HDAC inhibition prevents NF-κB activation by suppressing proteasome activity: down-regulation of proteasome subunit expression stabilizes I κ B α. *Biochem. Pharmacol.* **70,** 394–406.
37. Imre, G., Gekeler, V., Leja, A., Beckers, T., Boehm, M. (2006) Histone deacetylase inhibitors suppress the inducibility of nuclear factor-κB by tumor necrosis factor-α receptor-1 down-regulation. *Cancer Res.* **66,** 5409–5418.
38. Yin, L., Laevsky, G., Giardina, C. (2001) Butyrate suppression of colonocyte NF-κB activation and cellular proteasome activity. *J. Biol. Chem.* **276,** 44641–44646.
39. Lanier, L. L. (2008) Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* **9,** 495–502.
40. Vivier, E., Tomasello, E., Baratin, M., Walzer, T., Ugolini, S. (2008) Functions of natural killer cells. *Nat. Immunol.* **9,** 503–510.
41. Epling-Burnette, P. K., Bai, F., Painter, J. S., Rollison, D. E., Salih, H. R., Krusch, M., Zou, J., Ku, E., Zhong, B., Boulware, D., Moscinski, L., Wei, S., Djeu, J. Y., List, A. F. (2007) Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors. *Blood* **109,** 4816–4824.
42. Ogbomo, H., Michaelis, M., Kreuter, J., Doerr, H. W., Cinatl Jr., J., (2007) Histone deacetylase inhibitors suppress natural killer cell cytolytic activity. *FEBS Lett.* **581,** 1317–1322.
43. Schmudde, M., Friebe, E., Sonnemann, J., Beck, J. F., Broker, B. M. (2010) Histone deacetylase inhibitors prevent activation of tumour-reactive NK cells and T cells but do not interfere with their cytolytic effector functions. *Cancer Lett.* **295,** 173–181.
44. Ferlazzo, G., Pack, M., Thomas, D., Paludan, C., Schmid, D., Strowig, T., Bougras, G., Muller, W. A., Moretta, L., Munz, C. (2004) Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc. Natl. Acad. Sci. USA* **101,** 16606–16611.
45. Martin-Fontecha, A., Thomsen, L. L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., Sallusto, F. (2004) Induced recruitment of NK cells to lymph nodes provides IFN-γ for T(H)1 priming. *Nat. Immunol.* **5,** 1260–1265.
46. Elboim, M., Gazit, R., Gur, C., Ghadially, H., Betser-Cohen, G., Mandelboim, O. (2010) Tumor immunoediting by NKp46. *J. Immunol.* **184,** 5637–5644.
47. Guerra, N., Tan, Y. X., Joncker, N. T., Choy, A., Gallardo, F., Xiong, N., Knoblaugh, S., Cado, D., Greenberg, N. M., Raulet, D. H. (2008) NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* **28,** 571–580.
48. Halfteck, G. G., Elboim, M., Gur, C., Achdout, H., Ghadially, H., Mandelboim, O. (2009) Enhanced in vivo growth of lymphoma tumors in the absence of the NK-activating receptor NKp46/NCR1. *J. Immunol.* **182,** 2221–2230.
49. Smyth, M. J., Swann, J., Cretney, E., Zerafa, N., Yokoyama, W. M., Hayakawa, Y. (2005) NKG2D function protects the host from tumor initiation. *J. Exp. Med.* **202,** 583–588.
50. Imai, K., Matsuyama, S., Miyake, S., Suga, K., Nakachi, K. (2000) Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* **356,** 1795–1799.

KEY WORDS:
NKG2D · NKp46 · cytokines · cytotoxicity