Clinical & Translational Immunology

Australian and New Zealand Society for Immunology Inc.

Clinical & Translational Immunology 2024; e1500. doi: 10.1002/cti2.1500
www.wileyonlinelibrary.com/journal/cti

ORIGINAL ARTICLE

Synergistic integration of histone deacetylase inhibitors apparently enhances the cytokine-induced killer cell efficiency in multiple myeloma via the NKG2D pathway

Jingjing Pu[1], Amit Sharma[1], Ting Liu[2], Jian Hou[3] & Ingo GH Schmidt-Wolf[1]

[1]Department of Integrated Oncology, Center for Integrated Oncology (CIO) Bonn, University Hospital Bonn, Bonn, Germany
[2]Translational Biogerontology Lab, German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany
[3]Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

Correspondence
IGH Schmidt-Wolf, Department of Integrated Oncology, Center for Integrated Oncology (CIO) Bonn, University Hospital Bonn, Venusberg-Campus 1, Bonn 53127, Germany.
E-mail: ingo.schmidt-wolf@ukbonn.de

Received 16 February 2024;
Revised 12 March 2024;
Accepted 13 March 2024

doi: 10.1002/cti2.1500

Clinical & Translational Immunology
2024; **13**: e1500

Abstract

Objectives. The rapid recognition of epigenetic manipulation's potential in restricting cancer cell capabilities spurred translational initiatives, including histone deacetylase inhibitors (HDACis). Clinical trials on multiple myeloma (MM) demonstrated substantial benefits of HDACis, coupled with promising outcomes from cytokine-induced killer cell (CIK) immunotherapy. Intriguingly, the unexplored synergy of HDACis and CIK cell immunotherapy in MM prompted our study. **Methods.** We examined clinically relevant HDACis (panobinostat/LBH589 and romidepsin) alongside CIK cells derived from peripheral blood mononuclear cells across diverse MM cell lines (U266, RPMI8226, OPM-2 and NCI-H929). Utilising various *in vitro* methodologies, we investigated how HDACis enhance CIK cell lysis of myeloma cells through NKG2D/NKG2D ligand interactions. **Results.** The results of our analysis indicated several key findings. (1) Enhanced cytotoxicity of CIK cells in MM cells when combined with HDACis. (2) Significant increase in apoptosis, suggesting HDACis and CIK may together enhance apoptotic effects in specific MM cell lines. (3) Elevated IFN-γ secretion and alterations in granzyme B secretion because of the independent activity of HDACis. (4) Notably, HDACis increased the expression of MICA/B and ULBP2, crucial for inducing antitumor cytotoxicity of NKT cells. Validation through NKG2D receptor blocking in CIK cells with a purified mouse antihuman NKG2D antibody further supported our findings. **Conclusions.** Our analyses provide sufficient evidence to consider this clinically forgotten instance (HDACis-CIK cell combination) as a therapeutic priority for MM treatment. Furthermore, we suggest that NKG2D/NKG2D-ligand interactions activating NK/NKT cells may contribute to enhanced myeloma cell lysis in response to HDACis treatment by CIK cells.

Keywords: cytokine-induced killer cells, histone deacetylase inhibitors, immunotherapy, multiple myeloma, NKG2D

INTRODUCTION

Multiple myeloma (MM) is a clonal expansion of plasma cells in which genetic and epigenetic processes are significantly involved during the development and progression of this pathological condition. Despite recent advances in treatment modalities, MM remains incurable in most cases because of the drug resistance to all conventional therapies. Given that aberrant expression of epigenetic modifications has been observed in various cancers, MM is no exception to this observation.[1,2] Of particular interest in MM are variable patterns of histone deacetylase (HDACs), which have led to the exploration of several histone deacetylase inhibitors (HDACis)-based interventions *in vitro*, *in vivo* and in clinical trials over the last decades.[3–5]

Notably, the passive mechanisms underlying the involvement of epigenetic inhibitors, including HDACs, with non-oncological drugs in MM, have recently been discussed.[6] Recently, we have also revealed the mutual interactions between epigenetic machinery and non-coding genome in regulating gene expression by investigating the intriguing interactions between HDAC6-induced lncRNA and its potential sponge miRNA in MM.[7] HDACis have also been shown to sensitise NK cell-mediated killing by upregulating expression of Natural killer group 2 member D (NKG2D) ligands MICA/B or ULBP1 on cancer cells, thus suggesting that HDACis might have promising applications in cancer immunotherapy.[8,9] Like HDACis, cytokine-induced killer (CIK) cell immunotherapy has also been quite active in cancer.[10] Moreover, CIK cell immunotherapy already have a history of successful clinical trials in MM. For instance, Lin *et al*.[11] reported one case report of patient with multiple cancers (MM and lung cancer) concomitant with paraneoplastic dermatoses and demonstrated that after treatment with CIK cells, MM and lung cancer remained stable and concomitant paraneoplastic dermatoses improved markedly without side effects. Zhao *et al*.[12] also followed CIK cell therapy by examining 50 patients with MM ($n = 24$: chemotherapy, $n = 26$: combined chemotherapy with dendritic cell (DC/CIK) and confirmed a better immunomodulatory effect with the combination therapy. In 2017, Wang *et al*. published a meta-analysis including 12 trials with 594 MM patients and confirmed that adjuvant immunotherapy of dendritic cells-CIK cells enhanced the efficacy of chemotherapy for MM and further improved prognosis probably by reconstructing the immune function.[13]

Surprisingly, HDACis have never been tested in combination with CIK cell immunotherapy in the clinic. Being pioneers in CIK cell therapy, we previously raised the question whether CIK cells and HDACis would be compatible in MM. To address this, we tested several HDACis (e.g. sodium butyrate, valproic acid and trichostatin A) in combination with CIK cells (as the first preclinical model) and confirmed their significant impact on human MM cell lines (KMS-18 and U-266).[14] However, some questions remain unanswered, such as (1) what exactly are the mechanisms behind the synergistic effect of CIK cells with HDACis in MM and (2) whether genetic/gender differences could play a therapeutically restrictive role when testing HDACis-CIK combinations in clinics, because MM is known to occur more frequently in males compared with females. Considering this, herein, we extended our analysis by testing clinically applicable HDACis with CIK cells in genetically distinct MM cell lines. In addition, we investigated whether HDACis treatment could enhance CIK cell lysis of myeloma cells through NKG2D/NKG2D ligand interactions.

RESULTS

Phenotypic identification of CIK cells and the effect of HDACis on the viability of MM cell lines

In clinical trials, whether HDACis were used alone or in combination with other drugs, a plethora of knowledge exists about their relative success in MM (Figure 1 and Supplementary table 1).To advance the knowledge, we first confirmed the phenotypic identification of CIK cells and the effect of HDACis on the viability of MM cell lines. As the days went by, the proportion of $CD3^+CD56^+$ NKT cells (CIK cells) increased (Supplementary figure 1a, one representative data of three donors). To confirm this, we identified the heterogeneous population of peripheral blood mononuclear cells (PBMCs; Day 0) composed of $CD3^+CD56^+$ NKT cells ($7.0\% \pm 1.9\%$), $CD3^+CD56^-$ T cells ($56\% \pm 5.6\%$) and $CD3^-CD56^+$ NK cells ($11\% \pm 2.7\%$). After 13 days of *ex vivo* expansion, the bulk CIK cells were a heterogeneous population composed of $CD3^+CD56^+$ NKT cells ($27.0\% \pm 5.9\%$), $CD3^+CD56^-$ T cells ($71\% \pm 5.6\%$) and $CD3^-CD56^+$ NK cells

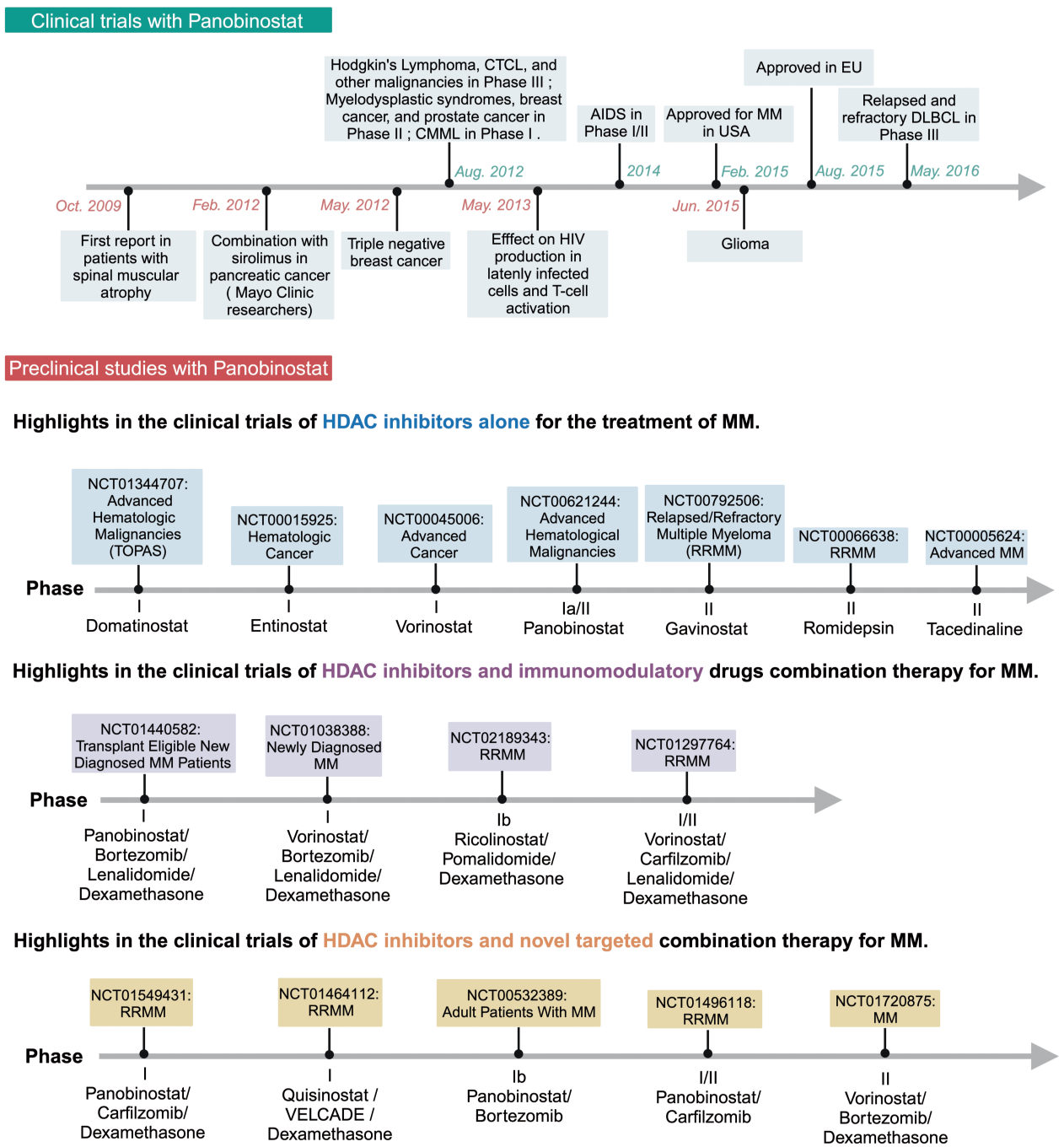

Figure 1. Histone deacetylase inhibitors (HDACis) used alone or in combination therapies in multiple myeloma. This figure was created with Biorender.com.

(0.63% ± 0.27%; Supplementary figure 1b). Likewise, the MM cell lines U266, RPMI8226, OPM-2 and NCI-H929 were cultured with gradient concentrations of panobinostat (0–250 nM) or romidepsin (0–10 000 nM) for 24 or 48 h (Supplementary figure 1c). As the concentrations of panobinostat and romidepsin increased, the viability of MM cells gradually decreased ($P < 0.05$),

and we also determined their IC_{50} values on MM cell lines. In order to avoid any background effect, we also tested effect of HDACis on viability of non-cancer cells (CCD-18Co) and CIK cells. CCD-18Co and CIK cells were treated with various concentrations of panobinostat and romidepsin. We observed that, at the appropriate concentration (for panobinostat, it is 0–50 nM; for romidepsin, it is 0–5 nM), the impact on the viability of MM cell lines was greater than that on the viability of CCD-18Co and CIK cells (Supplementary figure 1d). Overall, we established phenotypically distinct CIK cell populations and tested the cytotoxic effects of HDACis on MM cells.

Effect of CIK cells on MM cell lines and synergy of HDACis with CIK cells on MM cells

CIK cells were co-cultured with MM cell lines U266, RPMI8226, OPM-2, NCI-H929 and the control cell line CCD-18Co for 24 h (Figure 2a). CIK cells from two different buffy coats (Donor A and B) were used at different effector-to-target ratios (1:1, 5:1 and 10:1). As a result, when the ratio was 10:1, CIK cells significantly reduced the *in vitro* viability of MM cells ($P < 0.0001$). At a 5:1 ratio, CIK cells significantly reduced OPM-2, RPMI8226 and U266 *in vitro* viability ($P < 0.0001$), with the observed impact varying according to the different effector-to-target ratios. However, CIK cells also significantly decreased the viability of CCD-18Co when the ratio was 10:1 and 5:1 ($P < 0.0001$). In this context, the cytotoxic effect of CIK cells may be associated with the immune status of donors. To investigate a synergistic effect of panobinostat, romidepsin and CIK cells, myeloma cell lines NCI-H929, OPM-2 and U266 were cultured with panobinostat (0.01 μM), romidepsin (0.01 μM) and CIK cells at different effector-to-target ratios (10:1, 5:1, 1:1) for 24 h (Figure 2b). In NCI-H929, OPM-2 and U266 cell lines, we found that MM cells treated with panobinostat and CIK cells (10:1) showed more specific lysis compared with cells only treated with CIK cells (10:1) except NCI-H929 ($P < 0.0001$). In MM cells treated with romidepsin and CIK cells (10:1), a statistically significant increase in specific lysis was observed only in OPM-2 compared with cells treated only with CIK cells (10:1; $P < 0.0001$). Overall, it is reasonable to conclude the combination use of panobinostat and romidepsin with CIK cells may increase the specific lysis of MM cell lines.

Effect of HDACis and CIK cells on granzyme B and IFN-γ secretion in MM cells

Given that CIK cells are well established to release Granzyme B and IFN-γ as independent mechanisms to kill tumor cells, we evaluated their relative amounts in the supernatant using sandwich ELISA. In all three MM cell lines, Granzyme B secretion remained stable, regardless of HDACis treatment (Figure 3a). Similarly, CIK cells treated with HDACis exhibited increased total secretion. Significantly higher Granzyme B secretion was observed in the U266 cell line alone, following treatment with panobinostat or romidepsin, compared with co-culture with CIK cells alone (For panobinostat, $P < 0.05$; for romidepsin, $P < 0.01$). This suggests an increase in Granzyme B secretion in the U266 cell line following treatment with CIK cells, panobinostat and romidepsin. Likewise, MM cell lines were incubated with panobinostat (0.01 μM), romidepsin (0.01 μM) and CIK cells at the effector-to-target ratio (20:1) for 24 h. After 24 h, the supernatant was collected for a sandwich ELISA to evaluate IFN-γ secretion (Figure 3b). In all three MM cell lines, the basal secretion without any treatment was low. However, after treatment with romidepsin, there was significantly higher IFN-γ secretion in all three MM cell lines compared with only co-culture with CIK cells (U266, OPM-2: $P < 0.0001$; NCI-H929: $P < 0.05$). Meanwhile, after treatment with panobinostat, there was significantly higher IFN-γ secretion only in OPM-2 compared with only co-culture with CIK cells ($P < 0.0001$). This result suggests that after co-culture of MM cells, CIK cells, panobinostat and romidepsin treatment, IFN-γ secretion increased. Notably, after treatment with panobinostat (0.01 μM), romidepsin (0.01 μM) and CIK cells on the U266 cell line, we observed an increase in the overall number of apoptotic cells, particularly in the number of early apoptotic cells (Figure 3c). Furthermore, when compared with the untreated group (control), the proportion of early apoptotic cells and late apoptosis or necrosis cells significantly increased (Figure 3d; $P < 0.0001$). That is, CIK cells enhance the apoptosis of U266 cell line that have been treated with HDACis. This suggests that HDACis and CIK cells may together enhance apoptotic effects in specific MM cell lines.

Effect of HDACis on the NKG2D ligands in myeloma cells

As aforementioned, HDACis have been shown to sensitise NK cell-mediated killing by upregulating

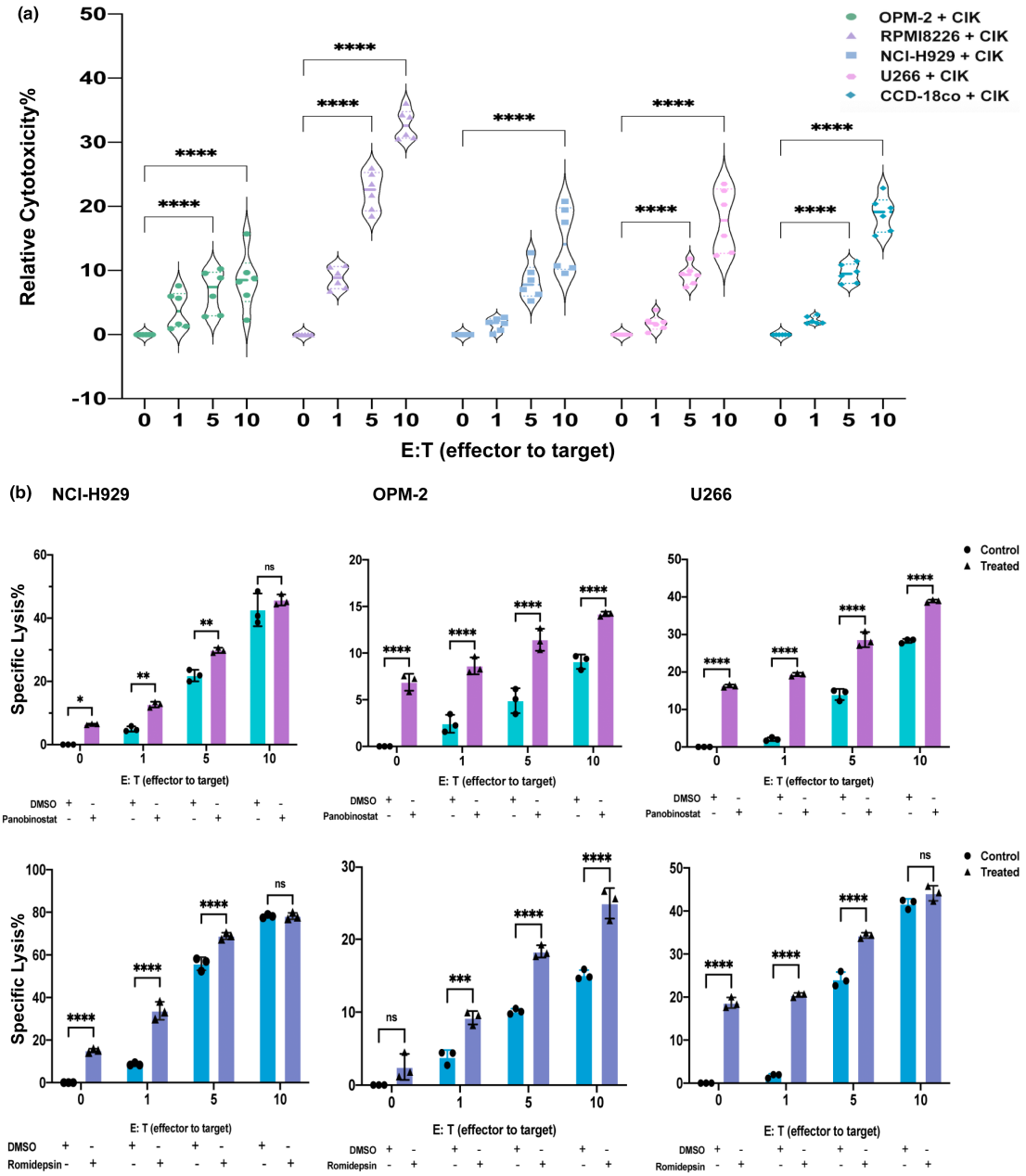

Figure 2. **(a)** Cytotoxic effect of cytokine-induced killer (CIK) cells on MM cell lines OPM-2, RPMI8226, NCI-H929, U266 and the control cell line CCD-18Co. Cells were cultured for 24 h at different effector: target ratios of 10:1, 5:1 and 1:1. The cytotoxic effect of CIK cells was measured by LDH assay. Results represent six separate experiments for U266, RPMI8226, OPM-2, NCI-H929 and CCD-18Co. **(b)** We assessed the significance among myeloma cells NCI-H929, OPM-2 and U266 in two conditions: one treated only with panobinostat (0.01 μM) and romidepsin (0.01 μM) for 24 h, and the other treated with the combination of panobinostat and romidepsin (at the same concentration). Additionally, CIK cells were treated at different effector: target ratios (10:1, 5:1 and 1:1). The results represent data from three separate experiments and are presented as mean ± SD. Significance levels were determined using two-way ANOVA with Bonferroni's post hoc test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

expression of NKG2D ligands MICA/B or ULBP1 on cancer cells. We investigated the effects of LBH589 and romidepsin on the mRNA expression of NKG2D ligands in myeloma cells. Specifically, we performed RT qPCR detection of NKG2D ligands in U266, OPM-2 and NCI-H929 before and after

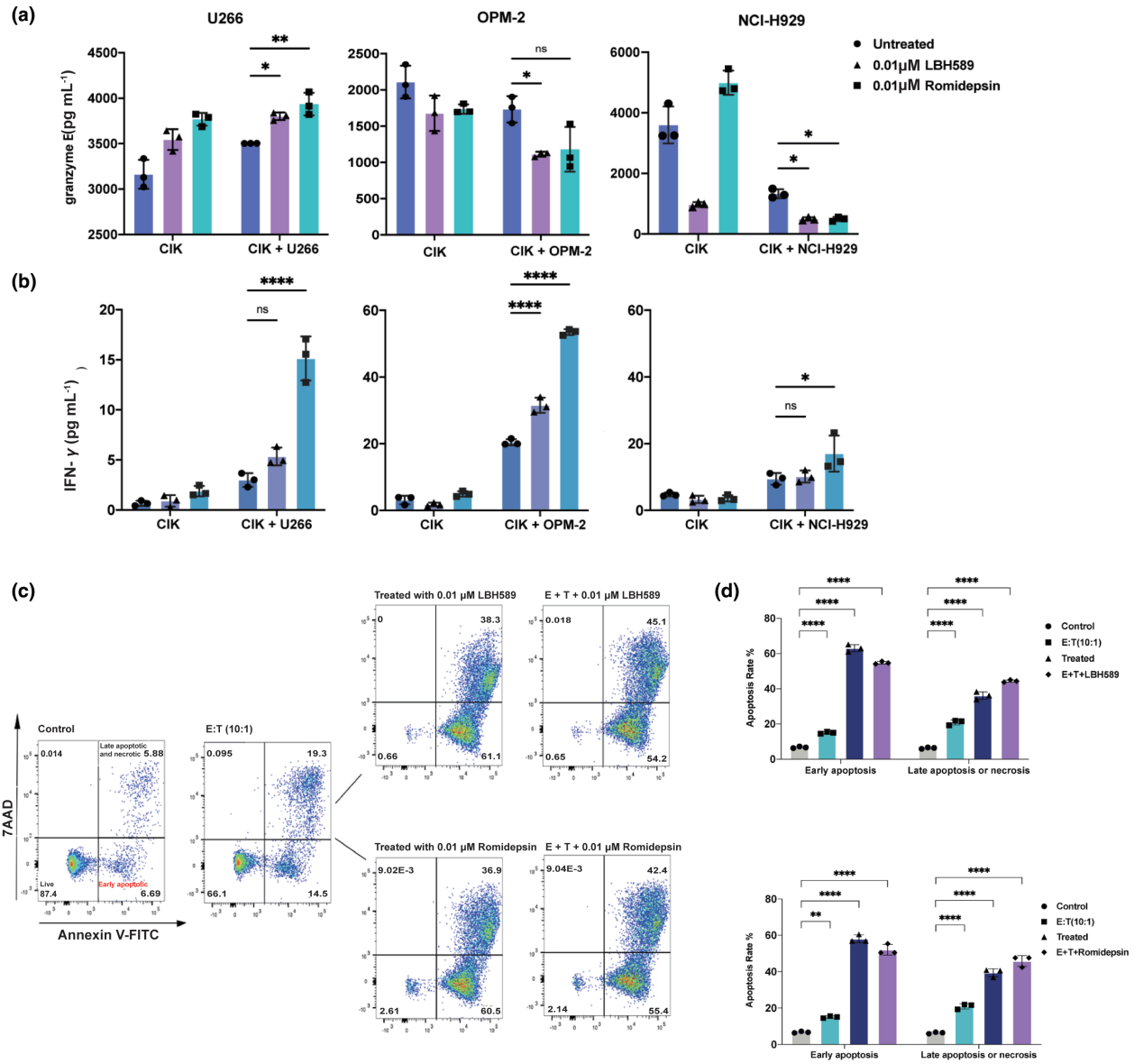

Figure 3. **(a)** Granzyme B secretion in the combination of histone deacetylase inhibitors (HDACis) and cytokine-induced killer (CIK) cells on multiple myeloma (MM) cell lines U266, OPM-2 and NCI-H929. **(b)** IFN-γ secretion in the combination of HDACis and CIK cells on MM cell lines. MM cells were cultured with panobinostat (0.01 μM), romidepsin (0.01 μM) and CIK cells at the effector-to-target ratio (20:1) for 24 h. IFN-γ and Granzyme B secretion were measured by ELISA. Results represent three different buffy coats for each cell line. **(c)** Combination of HDACis with CIK cells on the apoptosis of U266 cell line by the flow cytometry assay. Flow cytometry figure of changes in the proportion of early apoptosis cells and late apoptosis or necrosis cells. Cells were stained with FITC Annexin V and Percp 7AAD. The result is one of the representative data. **(d)** After the combination of HDACis with CIK cells, the apoptosis rate of U266 cells changed. Results represent data from three separate experiments. Data are presented as mean ± SD. (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ calculated by two-way ANOVA, Bonferroni's post hoc test).

treatment with LBH589 or romidepsin. We found that after LBH589 treatment, the relative mRNA levels of MICA, MICB and ULBP2 were significantly increased compared with the control group (Figure 4a, $P < 0.01$). Similar results were obtained after romidepsin treatment (Figure 4b, $P < 0.01$), suggesting that LBH589 and romidepsin can increase the expression of MICA, MICB and ULBP2 mRNA levels in MM cells. We further verified the effects of LBH589 and romidepsin on the surface

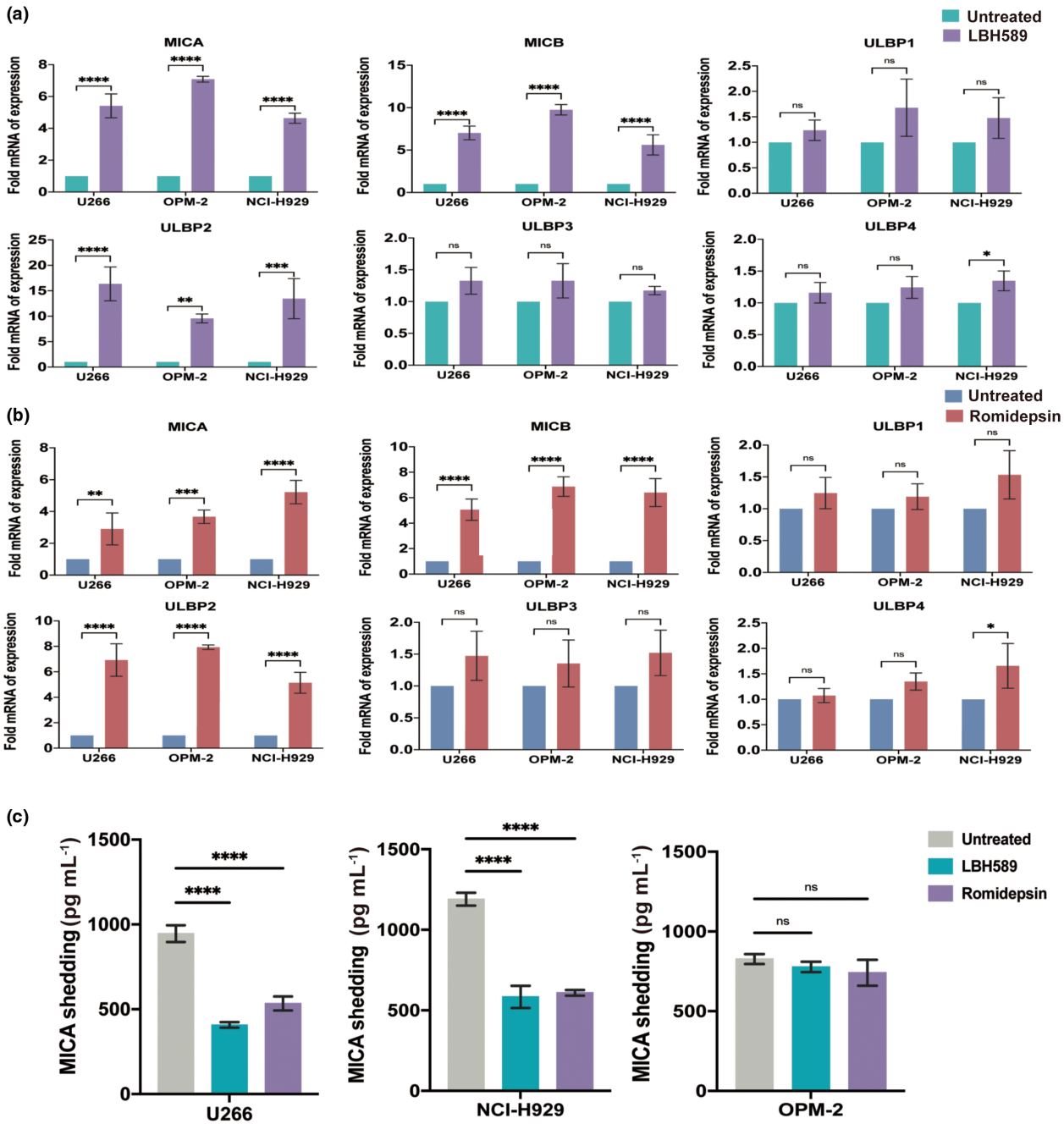

Figure 4. Relative mRNA expression of NKG2D ligands on human myeloma cell lines after treatment with 0.01 μM LBH589 or romidepsin for 24 h. **(a)** Relative mRNA expression of NKG2D ligands after LBH589 treatment on U266, OPM-2, NCI-H929 cells. **(b)** Relative mRNA expression of NKG2D ligands after romidepsin treatment on U266, OPM-2 and NCI-H929 cells. **(c)** Multiple myeloma (MM) cell lines (U266, OPM-2 and NCI-H929) were untreated or treated with 0.01 μM panobinostat or romidepsin for 48 h. Shed MICA was quantified in the supernatant by sandwich ELISA. Data are mean ± SD of triplicate measurements; data are one representative of three independent experiments. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ calculated by one-way ANOVA, Bonferroni's post hoc test).

expression of MICA/B and ULBP2 in myeloma cells by using flow cytometric analysis, gating on live U266, OPM-2 and NCI-H929 MM cells before and after treatment with LBH589 or romidepsin (Supplementary figure 2a). As depicted in Supplementary figure 2b, the treatment

significantly upregulated the expression of MICA/B ($P < 0.05$). With the exception of OPM-2 treated with LBH589, the expression of ULBP2 also significantly increased in the other cell lines after treatment ($P < 0.05$), suggesting that HDACis (LBH589 and romidepsin) can enhance the expression of NKG2D ligands (MICA/B and ULBP2). Furthermore, compared with the untreated group, the MICA shedding from U266 and NCI-H929 cells in the presence of 0.01 μM LBH589 or romidepsin was strongly inhibited (Figure 4c, $P < 0.0001$). Our findings indicate that LBH589 and romidepsin exhibit pronounced inhibitory effects on the proteolytic shedding of MICA from MICA/B-bearing tumor cells, leading to a significant augmentation in the cell surface density of MICA. Taken together, these results suggest that in MM, HDACis enhance the activation of the NKG2D pathway more efficiently.

HDACis treatment enhances CIK cell lysis of myeloma cells through NKG2D/NKG2D ligand interactions

Since the binding of MICA/B and ULBP2 ligands to NKG2D receptors causes antitumor cytotoxicity of NKT cells,[15] we investigated the functional relevance of increased MICA/B and ULBP expression in MM cells following HDACis therapy. Initially, we used a purified mouse antihuman NKG2D antibody to block the NKG2D receptor on CIK cells, employing $CD4^+$ T cells as a negative control group to assess the effectiveness of the blockade.[16] Subsequently, we observed that the NKG2D receptor on CIK cells was successfully blocked (Figure 5a). We examined CIK cells, isolated from healthy donors, against MM cells treated with or without HDACis, using the FACS cytotoxicity assay. We set up the lysis of NCI-H929 and U266 cell lines by CIK cells as the positive control (effector-to-target cell ratio – 5:1). MM cells treated with HDACis exhibited higher sensitivity to CIK cell lysis than untreated control cells. Furthermore, in the NCI-H929 cell line, only the enhancing effect of LBH589 was partially blocked by anti-NKG2D mAb pretreated CIK cells, which was statistically significant. In the U266 cell line, CIK cells pretreated with anti-NKG2D mAb significantly inhibited the potentiation by both LBH589 and romidepsin (Figure 5b). Our data suggest that the activation of NKT cells through NKG2D/NKG2D ligand interactions is a possible mechanism associated with the increased lysis of myeloma cells following HDACis treatment *in vitro* (Figure 5c).

DISCUSSION

The mechanisms of action of HDAC inhibitors, along with new data from preclinical experiments and clinical trials, have significantly broadened the range of cancers treatable with these compounds, especially MM. In clinical trials, whether HDACis were used alone or in combination therapies with other drugs, a plethora of knowledge exists about their relative success in MM. panobinostat, an orally administered HDACi, is also worth mentioning as it undoubtedly remains one of the best options for MM patients who require an additional therapeutic regimen, especially in relapsed or relapsed and refractory MM.[17] Nevertheless, the results of several preclinical evaluations with HDACis, including combination therapies in MM, suggest that they may be an interesting alternative to the established regimen.[3,18] The scenario is quite similar in case of CIK cell immunotherapy in the clinic,[10] which recently turned 30 years old and already has a history of successful clinical trials in MM. Surprisingly, HDACis have never been tested in combination with CIK cells in the clinical trials.

Herein, we tested clinically applicable HDACis (panobinostat/LBH589 and romidepsin) with CIK cells in genetically distinct MM cell lines (U266/male 53 years, RPMI8226/male 61 years, OPM-2/female 56 years, NCI-H929/female 62 years). The analysis showed panobinostat and CIK cells (effector-to-target cell ratio—10:1) increased specific lysis in all MM cell lines except NCI-H929. In contrast, the combination of romidepsin and CIK cells exerted this effect only in OPM-2. There was a significant increase in the proportion of cells with early apoptosis and late apoptosis or necrosis in U266 compared with the untreated group (control), suggesting that HDACis and CIK may together enhance apoptotic effects in specific MM cell lines. Romidepsin significantly increased IFN-γ secretion in all cell lines when used with CIK cells, whereas panobinostat showed this increase only in OPM-2. Likewise, changes in Granzyme B secretion were observed. Therefore, it is reasonable to speculate that intrinsic genetic/epigenetic factors may have a crucial role in a subset of patients when testing HDACis-CIK cells in the clinic. Since binding of

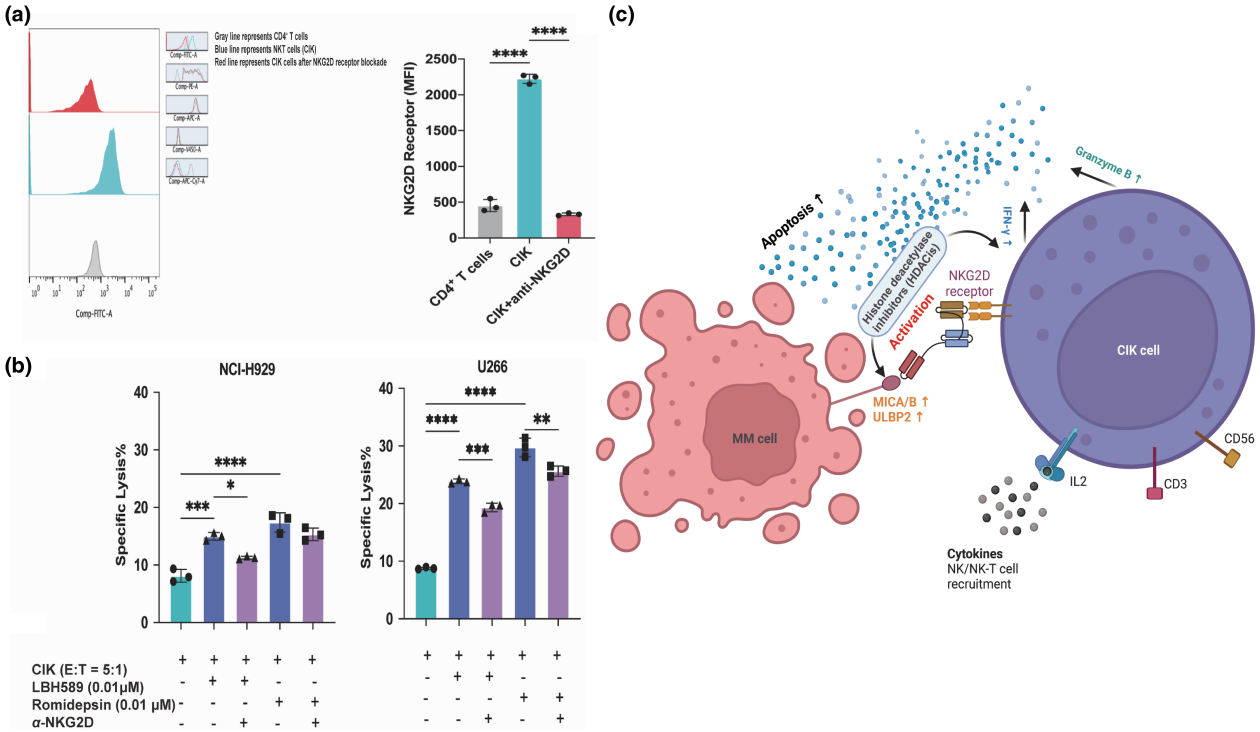

Figure 5. **(a)** Flow cytometry histogram of NKG2D receptor blocking experiment (grey line represents $CD4^+$ T cells, blue line represents cytokine-induced killer (CIK) cells, red line represents CIK cells after NKG2D receptor blockade), data are presented as mean ± SD. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ calculated by one-way ANOVA, Bonferroni's post hoc test). **(b)** CIK cells were incubated in the presence of anti-NKG2D Abs or medium alone for 60 min and then used for FACS cytotoxicity assay against histone deacetylase inhibitors (HDACis)-treated multiple myeloma (MM) cells at E/T 5:1. Data are presented as mean ± SD. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ calculated by two-way ANOVA, Bonferroni's post hoc test). **(c)** Schematic representation of a possible mechanism by which the effect of CIK cells may be enhanced by the addition of HDACis in MM. This schematic diagram was created with Biorender.com.

MICA/B and ULBP2 ligands to NKG2D receptors is well established to induce antitumor cytotoxicity of natural killer T cells,[15] we also investigated this axis and found that both HDAC inhibitors were able to significantly upregulate MICA/B and ULBP2 expression. Furthermore, we confirmed these results by blocking the NKG2D/NKG2D ligand interactions suggesting that it may be the activation of natural killer / natural killer T cells by NKG2D/NKG2D ligand interactions by which CIK cells potentiate enhanced lysis of myeloma cells in response to HDACis treatment. The evidence gleaned from these experiments strongly suggests a plausible mechanism underlying the observed phenomenon—namely the activation of natural killer and natural killer T cells via the NKG2D/NKG2D ligand interactions. This intricate cellular interplay is posited as the key contributor to the augmented lytic activity of CIK cells against myeloma cells following treatment with HDACis. The experimental design employed in this investigation not only validates the initial results but also advances our understanding of the intricate immunological processes at play during HDACis treatment. These insights contribute significantly to the broader comprehension of the cellular and molecular mechanisms orchestrating the anti-myeloma effects of CIK cells in response to HDACis, opening avenues for further exploration and therapeutic applications in the context of haematological malignancies. Since CIK cell immunotherapy has already showed promising results in MM clinical trials, in addition, approved in many countries, including Germany. Therefore, trials with CIK cells and certain inhibitors (e.g. HDACis) are quite feasible for MM patients. The same applies to HDACs for their modest efficacy as single agents in MM patients. Therefore, the

scenario for future MM research is quite straightforward. Beyond that, our supporting analyses reported here provide sufficient evidence to bring this clinically forgotten instance (HDACis-CIK cell combination) as a priority for MM treatment. The extent to which selective HDACi (e.g. ACY-1215/HDAC6) may be of benefit together with CIK cells compared with other non-selective HDACis could further help to refine treatment strategies in MM.

METHODS

Cell culture and HDACis

Genetically distinct MM cell lines (U266/ADM/male 53 years (RRID: CVCL_GZ72), RPMI8226/male 61 years (RRID: CVCL_0014), OPM-2/female 56 years (RRID: CVCL_1625), NCI-H929/female 62 years (RRID: CVCL_1600)) were used in this study. Cells were cultured in RPMI-1640 (Pan-Biotech, Aidenbach, Bavaria, Germany) medium supplemented with 10% FBS (Sigma-Aldrich Chemie GmbH, Munich, Germany) and 1% penicillin/streptomycin (Gibco, Schwerte, Germany) at 37°C, 5% CO_2, humidified atmosphere. All the cell lines were purchased from DSMZ (Braunschweig, Germany) and were mycoplasma-free, as tested by the mycoplasma detection kit (Thermo Fisher Scientific, Darmstadt, Germany). The control cell line CCD-18Co (RRID: CVCL_2379) (ATCC, Wesel, Germany; human colon fibroblasts) was cultured in Eagle's Minimum Essential Medium (ATCC) consisting of 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO_2. All the cell lines were authenticated by short tandem repeat profiling within 3 years prior to the research and regularly checked for mycoplasma contamination. Cells were cultured at 37°C, 5% CO_2.

Notably, panobinostat (LBH589, catalogue no. S1030, Selleck, Munich, Germany) and romidepsin (catalogue no. HY-15149, MedChemExpress), clinically relevant HDACis were used in the experiments. All drugs were used at different concentrations for 24–48 h.

Generation and phenotypic identification of CIK cells

For CIK cell generation, PBMCs were derived from buffy coats of healthy volunteers received from the Blutspendedienst at the University Hospital Bonn. We obtained approval from the ethics committee of the University Hospital Bonn, including signed informed consent from the volunteers. The CIK cells were cultured as described previously.[19] In brief, mononuclear cells were cultured in (10% FBS, 2.5% HEPES and 1% penicillin/streptomycin) RPMI-1640 with IFN-γ (ImmunoTools GmbH, Aidenbach) added on Day 0, 50 ng mL^{-1} of an antibody against CD3 (OKT, eBioscience, Thermo Fisher Scientific, Inc. San Diego, CA, USA), 100 U mL^{-1} IL-1β and 300 U mL^{-1} IL-2 (ImmunoTools GmbH, Aidenbach) were added 24 h later and cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and subcultured every 3 days in fresh complete medium containing IL-2 at 3×10^6 cells mL^{-1}. The CIK cells were harvested, and assays were performed at maturity after between 3 and 4 weeks of culture. At the end of expansion, CIK cells were harvested to determine phenotype by flow cytometry (FACS Canto II flow cytometer, BD Biosciences, Heidelberg, Germany). Cells were stained with FITC-CD3, FITC-NKG2D, APC-CD3, APC-CD56, PE-CD56, APC-Cy7-CD4 and corresponding isotype antibodies. The Hoechst 33258 dye (Cayman Chemical, Hamburg, Germany) was added before flow cytometry analysis to gate out intact viable cells. Samples were acquired using FACS Canto II flow cytometer.

CCK8 assay

Cells were seeded at a density of 5×10^3 to 10×10^4 per well in 96-well plates. They were then treated with varying concentrations of HDACis for 24–72 h before the addition of the Cell Counting Kit-8 (CCK-8) reagent. Following a further incubation period of 24–72 h, the plates were centrifuged for 4 min at 200 *g*, and 10 μL of CCK-8 reaction solution (product code: DJDB4000X, Dojindo Molecular Technologies, Inc.) was added. After 3–4 h, a colorimetric analysis was conducted using a microplate reader (Infinite® 200 PRO, Tecan) at 450 nm. The percentage inhibition of cell growth was calculated to determine the half-maximal inhibitory concentration (IC50) using Prism 5 (GraphPad Software, USA).

LDH assay

A commercial CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher, Waltham, MA, USA) was used according to the manufacturer's instructions. Effector cells (CI K cells) were co-cultured with target cells (MM cells) in 48-well plates at effector-to-target (E:T) ratios of 1:1, 5:1, 10:1. Then, the released LDH absorbance was measured using a microplate reader at 490 and 680 nm. At the end of incubation, 25 μL of each sample was transferred to a 96-well flat-bottom plate in different wells, and 25 μL of the reaction mixture was added to each well. To calculate LDH activity, subtract the absorbance value at 680 nm from that at 490 nm. All experiments were performed in triplicates. Experiments were replicated two times with CIK cells from two different donors. In order to calculate % cytotoxicity, the following equation was applied to the corrected values:

$$\%\ \text{Relative cytotoxicity} = (\text{Experimental value} - \text{Effector cells spontaneous control} - \text{Target cells spontaneous control}) / (\text{Target cells maximum control} - \text{Target cells spontaneous control}) \times 100$$

FACS cytotoxicity assay

For *in vitro* cytotoxicity assessment, a flow cytometry-based assay was performed as described previously[20] with some modifications. 4×10^6 tumor cells were labelled with 1.25 μM CFSE (Thermo Fisher Scientific, Eugene, USA) in 1 mL PBS for

20 min at 37°C in the dark. This was followed by two washes with 5 mL culture medium (containing 10% FBS) to remove excess CFSE dye. Next, an equal number of cells (5×10^4 per well) were co-cultured with CIK cells at different E:T ratios in 48-well round-bottom plate at 37°C, 5% CO_2. 0.01 μM drug was added into each well at the time of cytotoxicity assay. For NKG2D blocking experiments, CIK cells were incubated with purified mouse antihuman NKG2D antibody (clone 1D11, IgG1, Santa Cruz Biotechnology) or normal mouse control IgG (Santa Cruz Biotechnology) at 10 μg mL^{-1} 1 h before co-culture with tumor cells. After 20 h of co-incubation, cells were stained with the Hoechst 33258 dye and measured by FACS Canto II (BD). At least 10 000 CFSE-labelled tumor cells were collected in each sample. The following formula was employed for cytotoxicity calculation:

$$\text{Specific lysis } (\%) = \left(\frac{\text{CT}-\text{TE}}{\text{CT}}\right) \text{X } 100,$$

where CT is the absolute number of live CFSE-labelled tumor cells in control tubes (target cells alone) and TE is the absolute number of live CFSE–labelled tumor cells in test tubes (target cells and effector cells).

Enzyme-linked immunosorbent assay (ELISA)

IFN-γ production by CIK cells

CIK cells were co-cultured with myeloma cells (5×10^4) at a ratio of 20:1 for 24 h in 96-well flat-bottom plates, either without treatment or in the presence of 0.01 μM panobinostat or romidepsin. At the end of the culture period, the plates were centrifuged for 5 min at 300 *g*. A volume of 100 μL of cell-free supernatant were collected for Elisa assay using the IFN-γ kit from Invitrogen (Camarillo, CA, USA), following the manufacturer's instructions. Absorbance was measured with an ELISA reader (Infinite® 200 PRO, Tecan) at 450 and 570 nm wavelengths.

Granzyme B production by CIK cells

The co-culture method of tumor cells and CIK cells was the same as before. After 24 h of culture, the cell-free supernatant was harvested, and the level of granzyme B was determined using the Human Granzyme B Elisa kit (Duoset DY2906-05 and DY008, R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's instructions. Absorbance was measured with an ELISA reader (Infinite® 200 PRO, Tecan) at wavelengths of 450 and 570 nm.

MICA shedding by MM cells

5×10^4 MM cells per well were cultured in 48-well plates with 200 μL complete medium without treatment or in the presence of 0.01 μM panobinostat or romidepsin. After 48 h of culture, cell-free supernatant was harvested and the level of soluble MICA was determined using MICA Elisa kit (Duoset DY1300 and DY008, R&D Systems, Inc. Minneapolis, MN, USA) following the manufacturer's instruction. Absorbance was measured with an ELISA reader (Infinite® 200 PRO, Tecan) at 450 and 570 nm wavelengths.

Flow cytometry assay on apoptosis

To investigate the combination of HDACis with CIK cells on the apoptosis of the U266 cell line, 4×10^6 tumor cells were labelled with 1.25 μM Violet dye (Thermo Fisher Scientific, Eugene, USA) in 1 mL PBS for 20 min at 37°C in the dark. This was followed by two washes with 5 mL culture medium (containing 10% FBS) to remove excess Violet dye. Subsequently, a common number of cells (5×10^4 per well) were co-cultured with CIK cells at the E:T ratio of 20:1 in a 48-well round-bottom plate at 37°C with 5% CO_2. panobinostat or romidepsin at a concentration of 0.01 μM was added to each well. After 20 h of co-incubation, cells were stained with the FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, catalogue no: 640922) and measured by FACS Canto II. At least 10 000 Violet-labelled tumor cells were collected in each sample.

RT qPCR detection of NKG2D ligands RNA expression level

RNA extraction and quantitative polymerase chain reaction (qPCR) are used for isolating RNA from biological samples and amplifying specific RNA sequences to analyze gene expression levels, respectively. RNA isolation of MM cell line (OPM-2, U266 and NCI-H929) samples was performed with RNeasy Plus Mini Kit (QIAGEN, Germany, catalogue no: 74136). Complementary DNA was synthesised by reverse transcription using HiFiScript Kit (Invitrogen, USA). Quantitative polymerase chain reaction was performed on NKG2D ligands (MICA, MICB, ULBP1, ULBP2, ULBP3 and ULBP4) using P PowerTrack™ SYBR Green Mastermix (Thermo Fisher Scientific, catalogue no: A46109). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the internal reference gene. The primer sequences are shown in Supplementary table 2. The Δ–Δ Ct ($2^{-\Delta\Delta Ct}$) approach was used to measure the relative expression levels of target genes, which were standardised against GAPDH mRNA levels, respectively, for NKG2D ligands mRNA expression.

Surface expression of NKG2D ligands on MM cells

MM cells (1×10^5 per well) were cultured in 96-well round-bottom plates at 37°C, 5% CO_2. For co-culture, 0.01 μM panobinostat or romidepsin was added to each well. After 24 h of culture, MICA/B and ULBP2 on the cell surface were detected following staining with PE-conjugated anti-MICA/B antibody and PE-conjugated anti-ULBP2 antibody (R&D systems). Prior to the staining process, Fc receptors were blocked with human TrueStain FcX™ (BioLegend, Koblenz, Germany) at a final dilution of 1:100. The Hoechst 33258 dye was added before flow cytometry analysis for viable cells gating. Samples were acquired using FACS Canto II (a flow cytometry system).

Statistical analysis

FACS data were analysed using FlowJo V10.4 software (LLC, Ashland, Oregon, USA). Statistical analysis was performed using GraphPad Prism (version 8.0). Experimental data are presented as means ± SD. One-way or two-way analysis of variance (ANOVA) with Bonferroni's post hoc test was performed to analyse statistical significance. $P < 0.05$ was considered to be statistically significant.

ACKNOWLEDGMENTS

This work was supported by the Open Access Publication Fund of the University of Bonn. The CIO Aachen Bonn Köln Düsseldorf is kindly supported by the Deutsche Krebshilfe. JJP and TL are supported by the China Scholarship Council (CSC) from the Ministry of Education, China. Open Access funding enabled and organized by Projekt DEAL.

AUTHOR CONTRIBUTIONS

Jingjing Pu: Conceptualization; funding acquisition; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Amit Sharma:** Conceptualization; funding acquisition; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Ting Liu:** Conceptualization; investigation; methodology; visualization; writing – review and editing. **Jian Hou:** Conceptualization; resources; writing – review and editing. **Ingo GH Schmidt-Wolf:** Conceptualization; funding acquisition; resources; supervision; writing – review and editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions in the paperware present in the paper and/or the Supporting Information. The data sets generated during and/or analysed during the current study are available from the corresponding author on request.

REFERENCES

1. Mithraprabhu S, Kalff A, Chow A, Khong T, Spencer A. Dysregulated class I histone deacetylases are indicators of poor prognosis in multiple myeloma. *Epigenetics* 2014; **9**: 1511–1520.
2. Sharma A, Liu H, Herwig-Carl MC, Chand Dakal T, Schmidt-Wolf IGH. Epigenetic regulatory enzymes: Mutation prevalence and coexistence in cancers. *Cancer Investig* 2021; **39**: 257–273.
3. Ferro A, Pantazaka E, Athanassopoulos CM, Cuendet M. Histone deacetylase-based dual targeted inhibition in multiple myeloma. *Med Res Rev* 2023; **43**: 2177–2236.
4. Imai Y, Hirano M, Kobayashi M, Futami M, Tojo A. HDAC inhibitors exert anti-myeloma effects through multiple modes of action. *Cancers (Basel)* 2019; **11**: 475.
5. Peng X, Sun Z, Kuang P, Chen J. Recent progress on HDAC inhibitors with dual targeting capabilities for cancer treatment. *Eur J Med Chem* 2020; **208**: 112831.
6. Wang Y, Sharma A, Ge F *et al*. Non-oncology drug (meticrane) shows anti-cancer ability in synergy with epigenetic inhibitors and appears to be involved passively in targeting cancer cells. *Front Oncol* 2023; **13**: 1157366.
7. Pu J, Liu T, Sharma A, Schmidt-Wolf IGH. Balancing the interplay of histone deacetylases and non-coding genomes: A step closer to understand the landscape of cancer treatment. *BMC Med Genet* 2023; **16**: 295.
8. Zhu S, Denman CJ, Cobanoglu ZS *et al*. 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.
9. Shi P, Yin T, Zhou F, Cui P, Gou S, Wang C. Valproic acid sensitizes pancreatic cancer cells to natural killer cell-mediated lysis by upregulating MICA and MICB via the PI3K/Akt signaling pathway. *BMC Cancer* 2014; **14**: 370.
10. Sharma A, Schmidt-Wolf IGH. 30 years of CIK cell therapy: Recapitulating the key breakthroughs and future perspective. *J Exp Clin Cancer Res* 2021; **40**: 388.
11. Lin J, Zhu H, Lu X *et al*. Autologous cytokine-induced killer cells in the treatment of multiple myeloma concomitant with lung cancer and paraneoplastic dermatoses. *Intern Med* 2010; **49**: 2341–2346.
12. Zhao X, Ji CY, Liu GQ *et al*. Immunomodulatory effect of DC/CIK combined with chemotherapy in multiple myeloma and the clinical efficacy. *Int J Clin Exp Pathol* 2015; **8**: 13146–13155.
13. Wang Y, Lv B, Li K, Zhang A, Liu H. Adjuvant immunotherapy of dendritic cells and cytokine-induced killer cells is safe and enhances chemotherapy efficacy for multiple myeloma in China: A meta-analysis of clinical trials. *Drug Des Devel Ther* 2017; **11**: 3245–3256.
14. Stephan D, Weiher H, Schmidt-Wolf IGH. CIK cells and HDAC inhibitors in multiple myeloma. *Int J Mol Sci* 2017; **18**: 945.
15. Pievani A, Borleri G, Pende D *et al*. Dual-functional capability of $CD3^+CD56^+$ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. *Blood* 2011; **118**: 3301–3310.
16. Maccalli C, Scaramuzza S, Parmiani G. TNK cells ($NKG2D^+$ $CD8^+$ or $CD4^+$ T lymphocytes) in the control of human tumors. *Cancer Immunol Immunother* 2009; **58**: 801–808.
17. San-Miguel JF, Hungria VT, Yoon SS *et al*. Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: A multicentre, randomised, double-blind phase 3 trial. *Lancet Oncol* 2014; **15**: 1195–1206.

18. Zhou YB, Zhang YM, Huang HH *et al*. Pharmacodynamic, pharmacokinetic, and phase 1a study of bisthianostat, a novel histone deacetylase inhibitor, for the treatment of relapsed or refractory multiple myeloma. *Acta Pharmacol Sin* 2022; **43**: 1091–1099.
19. Schmidt-Wolf IG, Negrin RS, Kiem HP, Blume KG, Weissman IL. Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. *J Exp Med* 1991; **174**: 139–149.
20. Lorenzo-Herrero S, Sordo-Bahamonde C, Gonzalez S, Lopez-Soto A. A flow cytometric NK cell-mediated cytotoxicity assay to evaluate anticancer immune responses in vitro. *Methods Mol Biol* 2019; **1884**: 131–139.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.