

In vivo administration of histone deacetylase inhibitors does not impair natural killer cell function in HIV+ individuals

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Objective: Histone deacetylase inhibitors (HDACi) have proven to induce HIV-RNA and antigen expression in resting CD4⁺ T cells of antiretroviral therapy (ART)-treated HIV-infected individuals. However, to achieve viral eradication, immune clearance must follow latency reversal, and thus it is essential to understand the impact of latency reversal agents on immune function.

Design: Here we evaluate the impact of in-vivo administration of vorinostat (VOR) and panobinostat (PNB) during clinical trials on natural killer (NK) cell function and phenotype.

Methods: Cryopreserved peripheral blood mononuclear cells from HIV-positive participants receiving VOR (NCT01319383) or PNB (NCT01680094) were selected to assess the impact of the drugs on cell composition, activation, NK cell phenotype (CD16, NKG2D, NKp30, NKp46 and DNAM-1), cytotoxic activity (CD107a), and interferon (IFN)- γ production.

Results: No impairment of NK cell function was observed during treatment with either VOR or PNB. An increase in the frequency of CD3⁻CD56⁺ NK cells was consistently observed. Interestingly, after VOR administration, NK cells increased expression of NKp46 and CD16, and showed improved degranulation and IFN- γ production capacity. Moreover, taking together VOR and PNB samples, HIV DNA levels in CD4⁺ cells were negatively correlated with NK cell frequency and NK cell expression of CD16.

Conclusions: In-vivo treatment with HDACi does not have measurable negative effects on NK cell function, with some evidence of improved function *in vitro*. These results have important implications for potential combinatorial approaches to target HIV reservoirs, suggesting that the use of HDACis as a latency reversal agent could be paired with interventions to enhance NK cell activity or recruitment.

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Keywords: histone deacetylase inhibitors, HIV, natural killer cells, panobinostat, PNB, SAHA, VOR, vorinostat

Introduction

Research strategies geared towards curing persistent HIV infection have entered clinical testing [1]. The most

advanced of these approaches involves the administration of compounds known as latency-reversing agents (LRAs) to induce HIV gene transcription, thus allowing expression of latent virus within infected cells. Histone

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deacetylase inhibitors (HDACi) are drugs employed to treat certain cancers that have also been demonstrated to reactivate HIV from latency [2–6]. Vorinostat (SAHA, VOR) was the first drug from this family to be tested in the clinic as an LRA [7–10]. Following VOR, two other HDACi – panobinostat (PNB) [11] and romidepsin (RMD) [12,13] – also entered clinical study. All three drugs increased cell-associated HIV RNA (ca-RNA) in CD4⁺ T cells from antiretroviral therapy (ART)-suppressed individuals, but failed to reduce the frequency of latently infected cells. Therefore, approaches to enhance immune responses following latency reversal are required to eliminate infected cells.

Initial efforts towards enhancement of immune responses were focused on HIV-specific CD8⁺ T cells [14–18], either through ex-vivo expansion or vaccination strategies. In-vitro studies have suggested that some LRAs might have a negative impact on the viability or function of CD8⁺ T cells [19–21], although this has not been confirmed *in vivo* [14]. More recently, there has been a growing interest in the exploration of natural killer (NK) cells as cytotoxic effectors in HIV-eradication strategies, as they have shown potential to be clinically exploited for HIV clearance [22]. Critical to those endeavors is the assessment of the effect of LRAs on NK cell function. To that end, we previously performed a study evaluating the effect of ex-vivo LRA exposure on NK cells [23]. In that ex-vivo study, we demonstrated that different compounds cause diverse effects on NK cells, with differences observed even within a given class of compounds. We observed that although VOR did not negatively affect NK cell function, PNB and RMD induced undesirable alterations [23]. However, given that the in-vivo milieu cannot be fully replicated by ex-vivo modeling, assessing the impact of in-vivo exposure to LRAs on NK cell is extremely important. In the present study, we report a direct assessment of NK cell function in cells obtained from HIV+ participants receiving VOR or PNB in clinical studies.

Methods

Study samples

Viably frozen peripheral blood mononuclear cells (PBMCs) were derived from participants included in

two clinical trials involving two different HDAC inhibitors: VOR and PNB. Basic characteristics of the clinical trials performed with HDACi in the context of HIV are summarized in Table 1.

The VOR samples were derived from five participants enrolled in the clinical trial NCT01319383 [24], which evaluated the effect of 22 cyclical doses of VOR over a period of 12–16 weeks. Briefly, 400 mg of VOR was administered daily Monday to Wednesday for 4 weeks, for a total of 11 doses. After a rest period, participants received another 4 weeks of VOR doses Monday to Wednesday to reach a total of 22 doses. PBMCs from the participants were obtained by leukapheresis at baseline, after the first 4-week cycle (on the morning after the 11th dose, ‘11 VOR-doses’) and at the end of the study (on the morning after the 22nd dose, ‘22 VOR-doses’), and viably frozen until use.

The PNB specimens were derived from the CLEAR study (NCT01680094) [11], in which 15 participants were administered 20 mg PNB three times per week (Monday, Wednesday, and Friday) every other week for 8 weeks. PBMCs were obtained 8 h after a PNB dose. In the present study, we included samples from five of these participants, with one sample before treatment (‘pre-PNB’), after 4 doses (‘4 doses’), after 10 doses (‘10 doses’), and 4 weeks after completing treatment (‘follow-up’).

Participants included in both trials were virally suppressed (<50 HIV RNA copies/ml) on ART for at least 2 years, and had CD4⁺ cell counts above 470 cells/ μ l.

Cell processing

Viably frozen PBMCs from each participant at different time points were thawed simultaneously and rested on culture for 24 h. An aliquot was used to analyze the frequency of each cell population (i.e. CD4⁺, CD8⁺, and NK cells), and assess proliferation and activation markers. A second aliquot was used to isolate NK cells (EasySep Human NK Cell negative selection Enrichment Kit, StemCell Technologies, Vancouver, British Columbia, Canada) to study expression of activating receptors and immune function. After isolation, the purity of NK cells was consistently above 85%.

Table 1. Summary of the clinical trials conducted with HDACi in the context of HIV infection.

	Clinical trial	#	Dose	Dosing	References
VOR	NCT01319383	8	400 mg	One dose	Archin <i>et al.</i> , 2012 [7]
	NCT01319383	5	400 mg	Three doses/week for 8 weeks	Archin <i>et al.</i> , 2014 [24]
	NCT01365065	20	400 mg	One daily for 14 days	Elliott <i>et al.</i> , 2014 [10]
	NCT01319383	16	400 mg	One dose 1 + 1 after 48 h 1 + 1 after 72 h 10 doses every 72 hours	Archin <i>et al.</i> , 2017 [9]
PNB	NCT01680094	15	20 mg	3 times/week every other week for 8 weeks	Rasmussen <i>et al.</i> , 2014 [11]
RMD	NTC02092116	6	5 mg/m ²	1 time/week for 3 weeks (intravenous)	Søgaard <i>et al.</i> , 2015 [12]

HDACi, histone deacetylase inhibitors; PNB, panobinostat; RMD, romidepsin; VOR, vorinostat

Analysis of cell phenotype

Peripheral blood mononuclear cells were stained with a panel of monoclonal antibodies to define cell populations. The antibody panel included CD3-PerCP (clone SK7, BD Biosciences, San Jose, California, USA), CD4-FITC (clone RPA-T4; BD Biosciences), CD8-AlexaFluor700 (clone SK1; Biolegend, San Diego, California, USA), and CD56-PE (clone NCAM16.2; BD Bioscience). Cell subsets were derived from the singlets gate within the lymphocyte population, and were classified as NK cells when they were CD3⁻CD56⁺, CD4⁺ T cells when they co-expressed CD3⁺ and CD4⁺, and CD8⁺ T cells when they were double positive for CD3⁺ and CD8⁺ (Fig. 1a). In addition, proliferation and activation markers were analyzed using Ki67-PE/Cy7 (Biolegend) and CD69-APC (clone FN50; Biolegend). Another antibody panel was designed to examine the expression of NK-activating receptors on isolated NK cells. This panel included the following antibodies from Biolegend: CD16-Alexa-Fluor700 (clone 3G8), NKG2D-APC (clone 1D11), NKp30-PE (clone P30-15), NKp46-PE/Cy7 (clone

9E2), and DNAM1-FITC (clone 11A8). Briefly, cells were resuspended in staining buffer and incubated with an optimized antibody concentration for 20 min on ice in the dark. Cells were then washed twice, fixed and analyzed using the Attune NxT Acoustic Focusing Cytometer (Applied Biosystems, Foster City, California, USA). Fluorescence minus one controls (FMO) were used for each antibody combination to set the gates. Analysis was performed using the FlowJo software v10 (Ashland, Oregon, USA).

Functional assays

The function of NK cells was assessed by measuring degranulation, through the degranulation marker CD107a [25], and interferon (IFN)- γ production by intracellular staining. Isolated NK cells were cocultured with the major histocompatibility complex class I lacking target cell line K562 at a 1:1 effector-to-target ratio, along with CD107a-PE/Cy7 (clone H4A3, Biolegend) for 2–4 h, adding the protein transport inhibitor Golgi-Stop (BD Biosciences) after the first hour. Cells were then

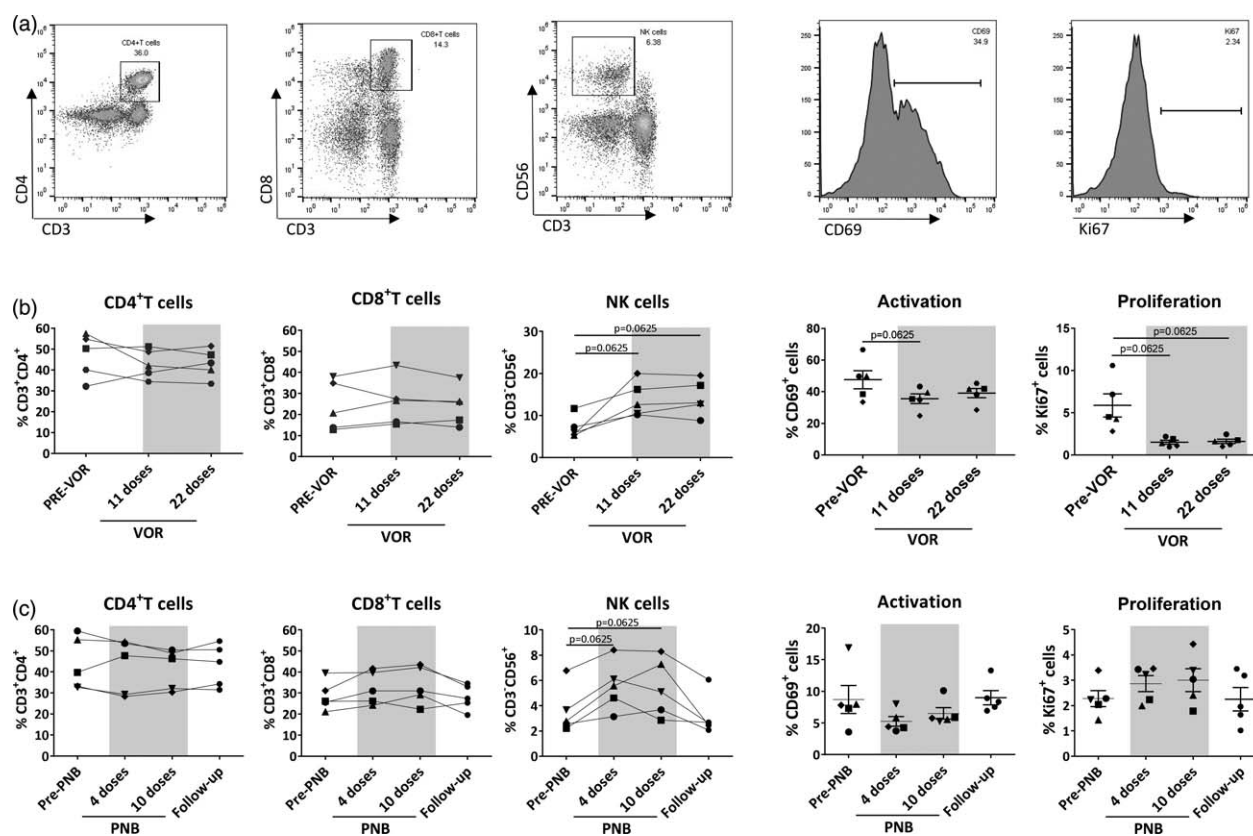


Fig. 1. Frequency of cell populations, proliferation, and activation during HDAC inhibitor treatment. The PBMCs were stained with a panel of monoclonal antibodies to analyze cell populations and expression of proliferation and activation markers. (a) Representative plot of cell gating strategy. (b) VOR treatment: the proportion of CD4⁺ and CD8⁺ T cells did not change during VOR treatment, but frequency of NK cells increased after 11 doses of VOR, with increases maintained after 22 doses. Expression of the proliferation marker Ki67 and the activation marker CD69 decreased during VOR treatment. (c) PNB treatment: CD4⁺ and CD8⁺ T cells remained stable during PNB treatment, but NK cells showed an increase, that was lost after the end of treatment. A decrease in CD69 was observed during PNB treatment. Each symbol represents cells from a different study participant. Wilcoxon matched-pairs signed rank test. HDAC, histone deacetylase; PBMCs, peripheral blood mononuclear cells; PNB, panobinostat; VOR, vorinostat.

harvested and stained with CD3-APC/Fire750 (clone SK7; Biolegend) and CD56-FITC (clone HCD56; Biolegend). Cells were washed, and after fixation and permeabilization, intracellular staining with IFN- γ -PE (clone 4SB3; Biolegend) antibody was performed. Cells were acquired using the Attune NxT Acoustic Focusing Cytometer, and analysis was performed using the Flowjo v.10. Cells were gated according to CD3 and CD56 expression to determine the proportion of NK cells expressing the degranulation marker CD107a and/or producing IFN- γ (Fig. 2a).

Frequency of latent HIV infection

Measurements of the frequency of latent HIV infection within each participant was determined at the different time points as described in detail by Archin *et al.* [24] for VOR, and Rasmussen *et al.* [11] for PNB. Briefly, measurement of the frequency of infection included quantification of HIV-1 *pol* DNA copies from resting or total CD4⁺ T cells, analyzed by digital droplet PCR (ddPCR), and quantification of replication-competent virus using the quantitative viral outgrowth assays (QVOA), which provides a minimum estimation of the frequency of replication competent HIV reported as infectious units per million cells (IUPM).

Ethics statement

All donors provided written informed consent. Studies were approved by the University of North Carolina Institutional Review Board for the VOR samples and by the Danish Research Ethics Committee system for PNB samples. Specimens used in the study were anonymized.

Statistical analysis

Given the limited sample size, the statistical analysis was exploratory with no adjustment for multiple testing. Pretreatment versus post-treatment measurements were compared with an exact two-sided Wilcoxon signed-rank test. For each study (VOR and PNB), measurements from five donors were available. Using a two-sided Wilcoxon signed-rank test, the smallest obtainable exact *P* value with *n* = 5 is *P* = 0.0625. Analysis was performed with GraphPad Prism v7. The correlation between the different parameters and the size of the reservoir was assessed by pooling measurements from pretreatment, mid-treatment, and post-treatment. A Spearman's *r* was calculated, but we also performed a Kendall tau correlation, because there were several measures from each participant.

Results

Natural killer cell frequency is increased after in-vivo administration of HDACi

For VOR, phenotypic analysis on PBMCs was performed at three time points: before VOR administration ('pre-VOR'), after 11 doses, and after 22 doses. Levels

of CD4⁺ and CD8⁺ T cells remained constant over the treatment period. However, we observed an increase in the frequency of NK cells in all donors, who had a mean of 7.13% NK cells at baseline, 13.9% after 11 doses, and 14.24% after 22 doses (Fig. 1b).

For PNB, analysis was performed in PBMCs at four time points: pre-PNB, after 4 and 10 doses, and after completion of treatment ('follow-up'). As with VOR, there was no change over time in the proportion of CD4⁺ or CD8⁺ T cells, but consistent with VOR, we observed an increase in the frequency of NK cells during PNB treatment in all participants (3.6% pre-PNB, 5.6% at visit 6, 5.4% at visit 10 and back to 3.2% in the follow-up sample; Fig. 1c).

Natural killer cells can be classified into bright and dim according to CD56 expression. Interestingly, we observed that CD56^{dim} NK cells proportion increased in all participants (median of 93.72% before treatment and 96.1% after drug exposure), whereas CD56^{bright} frequency decreased in most of them (median of 5.6 vs. 3.47%).

Expression of the proliferation marker Ki67 and the early activation marker CD69 were also analyzed in total PBMCs and within the different cell subsets. Both VOR and PNB treatment resulted in a decrease of the expression of CD69, either analyzing the entire cell population (Fig. 1b, c) or the different cell subsets (data not shown). Conversely, Ki67 expression pattern differed after treatment with VOR or PNB. Ki67 expression significantly decreased after VOR treatment (Fig. 1b), whereas it remained unchanged after PNB treatment (Fig. 1c).

In-vivo VOR treatment increases natural killer cell capacity to degranulate and produce interferon- γ

Natural killer cell degranulation and IFN- γ production potential were evaluated after coculture of isolated NK cells with the cell line K562. Degranulation was measured using surface CD107a expression, and IFN- γ production was assessed by intracellular staining. Due to limited sample availability, NK cell function could only be assessed in response to K562 cells, but we acknowledge that additional analysis of NK cell responses such as antibody-dependent cell cytotoxicity would provide further evidence of function alterations. Interestingly, VOR treatment improved both degranulation and IFN- γ production of NK cells after culture with K562 cells (Fig. 2b). Conversely, we did not find any function alteration after PNB treatment (Fig. 2c). In addition, we found a positive correlation between the frequency of NK cells, and both degranulation and IFN- γ production. Functional markers also correlated positively with the expression of NKp30, NKp46, NKG2D, and CD16 (data not shown).

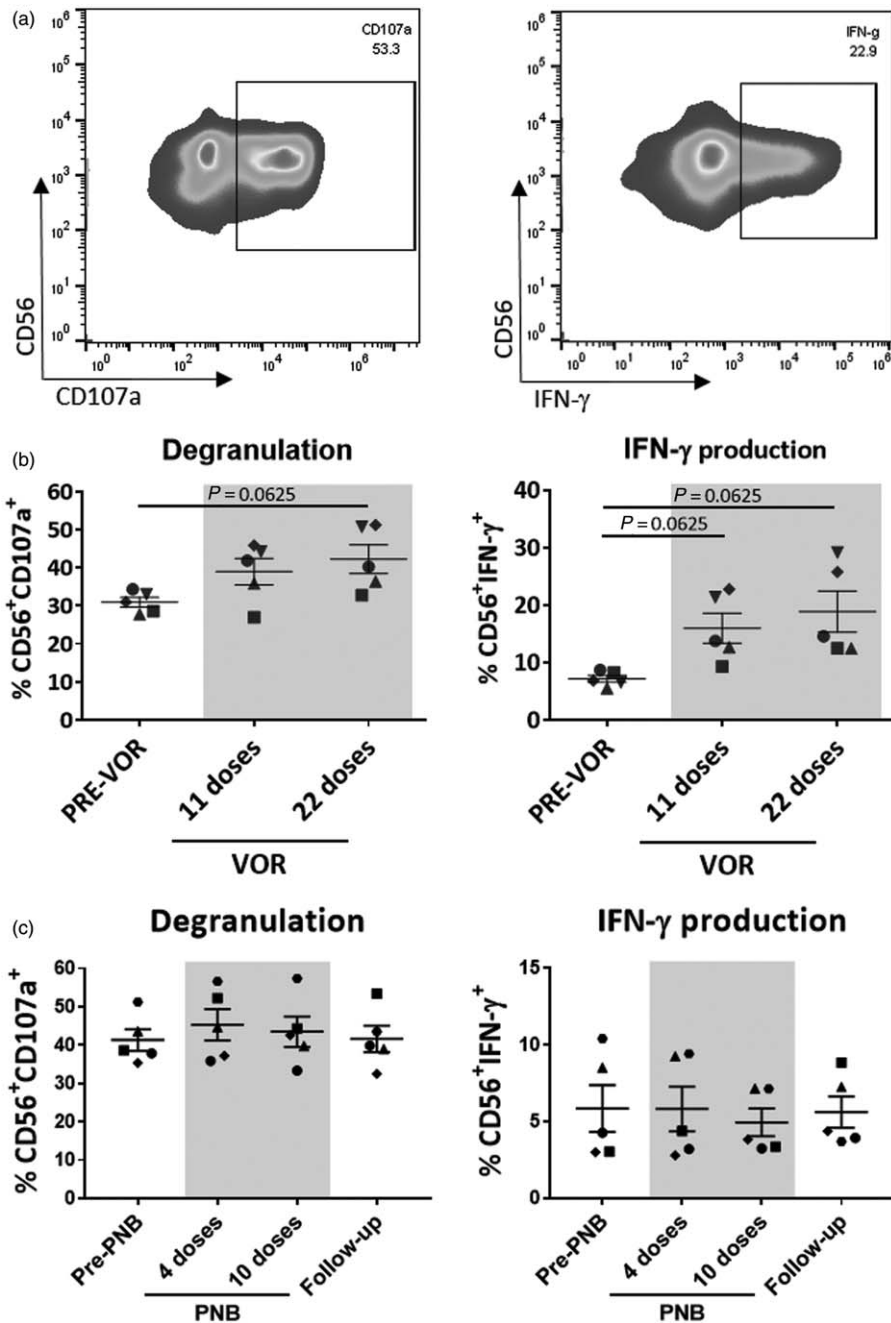


Fig. 2. Natural killer (NK) cell cytotoxicity and cytokine production. The NK cells were negatively isolated from thawed PBMCs and cultured with the cell line K562 to measure degranulation through the marker CD107a and IFN- γ production by intracellular staining. (a) Representative plots of cells from one participant. (b) VOR treatment: an increase in both degranulation and IFN- γ production was observed after VOR exposure. (c) PNB treatment: cytotoxic and IFN- γ potential remained stable during PNB treatment. Each symbol represents cells from a different study participant. Wilcoxon matched-pairs signed-rank test. IFN, interferon; PNB, panobinostat; VOR, vorinostat.

CD16 and NKp46 are up-regulated after vorinostat treatment

The NK cells express a wide variety of receptors that enable them to differentiate tumor or infected cells from healthy cells. These include inhibitory, activating,

adhesion, and cytokine receptors. The balance of these signals determines whether NK cells become activated or not [26]. Following NK cell isolation by negative selection, we analyzed the expression of a selection of activating receptors on the surface of NK cells. Expression

of DNAM-1, NKp30, and NKG2D were not altered by VOR exposure, but interestingly, NKp46 and CD16 expression were up-regulated to some extent in most participants (Fig. 3a). Specifically, CD16 up-regulation occurred exclusively within the CD56^{dim} subset, whereas it decreased in the CD56^{bright} population. In contrast, PNB treatment did not seem to impact in the expression of any receptor (Fig. 3b).

Correlation with the HIV reservoir size

We analyzed whether the different parameters measured in the present study were correlated with the size of the reservoir, measured either as total DNA in CD4⁺ cells or as IUPMs. Interestingly, after combining all time points from both VOR and PNB studies, some correlations were found. There was a negative correlation between the frequency of NK cells and the copies of DNA/million CD4⁺ cells (Spearman's $r = -0.51$, $P = 0.0017$, Kendall's tau = -0.33 , 95% CI $-0.57, -0.08$). Similarly, there was a negative correlation between CD16 expression in NK cells and HIV-DNA (Spearman's $r = -0.43$, $P = 0.01$, Kendall's tau = -3.8 , 95% CI $-0.72, 0.17$), and also a trend ($P = 0.09$) towards a negative correlation between NK cell IFN- γ production and HIV-DNA content. Unexpectedly, a positive correlation was observed between NKG2D expression and HIV-DNA (Spearman's $r = 0.35$, $P = 0.041$, Kendall's tau = 0.26 , 95% CI $-0.08, 0.52$) (Fig. 4). No significant correlation was found between HIV reservoir size measured as IUPM and any of the analyzed parameters (data not shown).

Discussion

One of the present strategies aimed at eradicating persistent HIV infection consists of combining LRAs with approaches to induce robust immune responses capable of identifying and clearing reactivated cells. Therefore, it is critical to discern if interventions used for latency reversal have any impact on the immune function needed for viral clearance. In the present study, we have evaluated the effect of in-vivo administration of HDACi on NK cell function. We have used samples from a clinical trial involving VOR (NCT01319383), in which five HIV-1-infected participants were administered 22 cyclical doses of 400 mg VOR over a period of 12 weeks [24], and a clinical trial involving PNB (NCT01680094) [11], in which 15 participants received 20 mg PNB three times a week every other week for 8 weeks. We analyzed the phenotype and the functionality of the immune cells in samples obtained before, during, and after clinical exposure to the drugs, focusing on NK cells.

The main finding of the study is that NK cell immune function was not impaired in samples from participants treated with either of the two HDACi. In addition, a moderate but consistent increase in the frequency of NK cells during HDACi treatment was observed, contrasting with stable frequencies of CD4⁺ and CD8⁺ T cells. Unfortunately, whether the observed increase in NK cells in peripheral blood is due to proliferation or to cellular redistribution, cannot be concluded from this study. Interestingly, we observed an enhancement in the

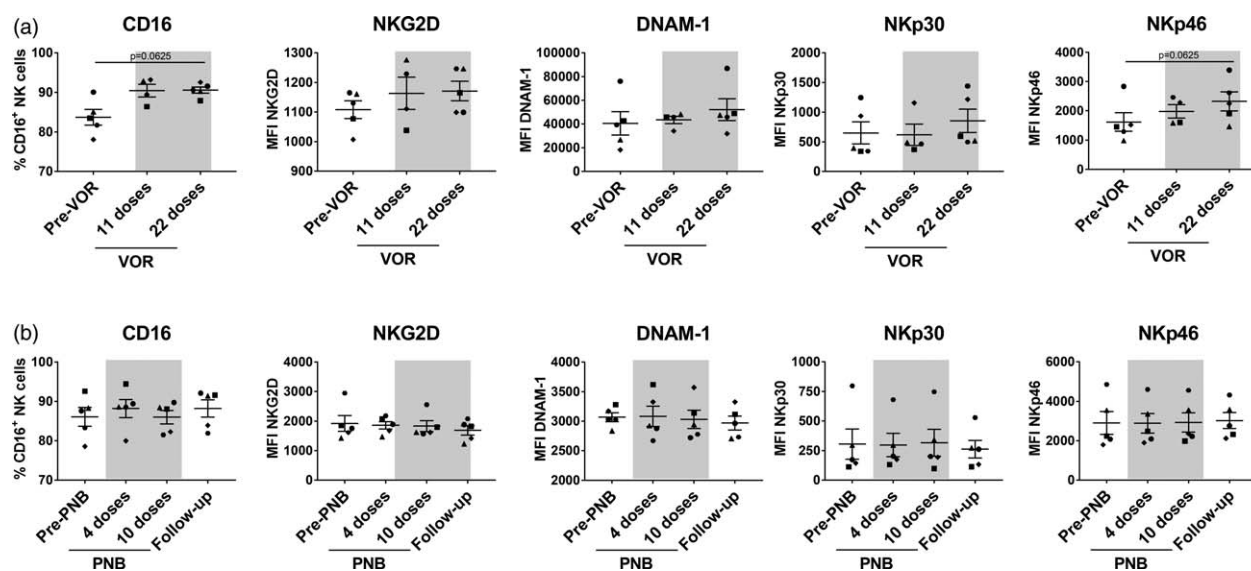


Fig. 3. Expression of activating receptors on natural killer (NK) cells. The NK cells were negatively isolated from thawed PBMCs and stained with a panel of monoclonal antibodies to analyze the expression of activating receptors. (a) VOR treatment: NKp30, DNAM-1, and NKG2D expression did not change during treatment, but expression of CD16 and NKp46 was increased to some extent after VOR administration. (b) PNB treatment: no changes were observed during PNB treatment. Each symbol represents cells from a different study participant. Wilcoxon matched-pairs signed-rank test. PBMCs, peripheral blood mononuclear cells; PNB, panobinostat; VOR, vorinostat.

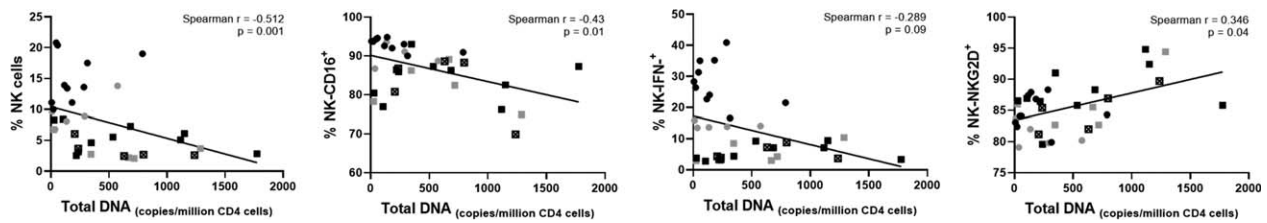


Fig. 4. Correlation with the HIV reservoir size. Size of the HIV reservoir was measured as total DNA per million CD4⁺ cells or as IUPMs after quantitative outgrowth assay. HIV DNA correlated negatively with NK cell frequency, CD16 expression, and IFN- γ production, whereas it correlated positively with NKG2D expression. Circles correspond to samples from the VOR study and squares to the PNB study. Grey represent samples before HDACi treatment, black during treatment, and crossed black after treatment. HDACi, histone deacetylase inhibitors; IUPM, infectious units per million cells.

capacity of NK cells to degranulate and produce IFN- γ following in-vivo administration of VOR. This improvement was accompanied by an increase in the expression of the activating receptors CD16 and NKP46, which, in addition, were positively correlated with degranulation and/or production of IFN- γ . PNB treatment did not cause any functional impairment, but no improvement was observed either. Finally, pooling together the data from both VOR and PNB studies, we observed an interesting negative correlation between the copies of HIV DNA/million CD4⁺ cells and the frequency of NK cells, and also with NK cell expression of CD16.

The question of whether HDACi suppress immune function is still under debate. Concern was initially raised when Jones *et al.* [19] reported that HDACi impaired cytotoxic T-lymphocyte-mediated IFN- γ production and the elimination of HIV-infected cells *in vitro*. However, later studies only partially confirmed these observations [15,20], highlighting the importance of factors such as specific HDACi classes, drug concentrations, and the length of drug exposure, which could have acted as confounding players in study outcomes.

This is the first evaluation of NK cell function performed after in-vivo administration of VOR or PNB. Previous studies have evaluated the impact of VOR exposure on NK cell function *in vitro* or *ex vivo*. We performed a comprehensive analysis of the effect of ex-vivo exposure of different HIV LRAs on NK cell phenotype and function. In regards to VOR, we did not observe any major impact on NK cells [23], in agreement with the present in-vivo results. However, other studies evaluating in-vitro impact of VOR exposure on NK cell parameters reported contradictory results. Pace *et al.* [27] reported similar results as our group, detecting no impairment of NK cell function after exposure to VOR, whereas Ogbomo *et al.* [28] and Pfeiffer *et al.* [29] observed a decrease in NK cytotoxicity. One potential explanation for these discrepancies is the different concentration and time of exposure used. In our experiments, and also in Pace *et al.*'s study, a concentration of 335 nmol/l was used over 24 h, to model peak VOR exposures *in vivo*, as VOR

is cleared in less than 6 h [30]. On the contrary, Ogbomo *et al.* used a continuous VOR concentration of 0.5–1 μ mol/l over 96 h, and Pfeiffer *et al.* 1 μ mol/l over 48 h. Regarding PNB, in our ex-vivo study, we observed that a PNB exposure consisting of 20 ng/ml during 24 h, which aimed to mimic physiologic conditions, increased NK cell death and reduced immune function [23], whereas in the present in-vivo study, we did not observe such impairment. Therefore, while in-vitro studies are undoubtedly of interest, it is obvious that in-vivo administration of the drug entails more complicated pharmacodynamics and cellular interactions that might provide different outcomes.

Studies of NK cell immune function after in-vivo exposure to HDACi are limited. In the clinical trial using PNB in HIV-1-infected individuals, Olesen *et al.* [31] found a correlation between HIV reservoir decline during PNB treatment and NK cell frequency, reporting that the proportions of total and CD56^{dim}CD16⁺ NK cells were inversely associated with HIV-1 DNA levels [31]. In agreement to these results, when we pooled the data obtained in the VOR and PNB study for sample size purposes, we observed a negative correlation between HIV DNA content and the proportion of NK cells (Fig. 4). However, we failed to detect such a correlation with the size of the replication competent reservoir (estimated as IUPMs by viral outgrowth assay), but this can be due to a reduced sample size, because IUPM data were available only in a subset of samples. In the present study, in addition to the correlation between HIV DNA and NK cell frequency, we observed an interesting negative correlation between HIV DNA and CD16 expression, and also a trend towards reduced HIV DNA content and NK cell IFN- γ production. Interestingly, this observation has been reported previously [32], and thus, the hypothesis that enhanced NK cell phenotype and function might have an impact in the HIV reservoir size deserves further investigation.

In summary, in-vivo administration of HDACi does not negatively impact NK cell function. Furthermore, we observed an increase in NK cell frequency during HDACi

treatment, and interestingly, VOR exposure improved cytotoxicity potential and capacity of NK cells to produce IFN- γ , and also the expression of the activating receptor NKp46. Finally, there was a negative correlation between the size of the HIV reservoir measured as HIV DNA in CD4⁺ T cells, and the frequency of NK cells and expression of CD16. These results have important implications in light of HIV-eradication strategies combining latency reversal with immune enhancement interventions.

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Author contributions: C.G. conceived and designed the study; D.M.M. and N.S.S. provided input in study design and experiment planning; C.G. and B.A. performed the experiments; D.M.M., N.M.A., M.T., T.A.R., and O.S.S. contributed reagents/materials; C.G. wrote the manuscript; and N.S.S., D.M.M., N.M.A., M.T., and O.S.S. reviewed it.

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Conflicts of interest

There are no conflicts of interest.

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