



Published in final edited form as:

AIDS. 2019 March 15; 33(4): 605–613. doi:10.1097/QAD.0000000000002112.

In Vivo Administration of Histone Deacetylase Inhibitors Does Not Impair Natural Killer Cell Function in HIV+ Individuals

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Abstract

Objective: Histone deacetylase inhibitors (HDACi) have proven to induce HIV-RNA and antigen expression in resting CD4⁺T cells of 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 PBMC 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 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

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CONFLICT OF INTEREST
None declared.

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 do 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.

Keywords

HIV; NK cells; Vorinostat; VOR; SAHA; Panobinostat; PNB; HDACi

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 deacetylase inhibitors (HDACi) are drugs employed to treat certain cancers that have also 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 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 while 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: vorinostat (VOR) and panobinostat (PNB). Basic characteristics of the clinical trials performed with HDACi in the context of HIV are summarized in Table 1.

VOR samples were derived from 5 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.

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 hours 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 antiretroviral therapy for at least 2 years, and had CD4 counts above 470 cells/uL.

Cell processing

Viably frozen PBMCs from each participant at different time points were thawed simultaneously and rested on culture for 24 hours. 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, BC) to study expression of activating receptors and immune function. After isolation, the purity of NK cells was consistently >85%.

Analysis of cell phenotype

PBMCs were stained with a panel of monoclonal antibodies to define cell populations. The antibody panel included CD3-PerCP (clone SK7, BD Biosciences), CD4-FITC (clone RPA-T4, BD Biosciences), CD8-AlexaFluor700 (clone SK1, Biolegend) 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 (San Diego, CA): CD16-AlexaFluor700 (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 minutes on ice in the dark. Cells were then washed twice, fixed and analyzed using the Attune NxT Acoustic Focusing Cytometer (Applied Biosystems, Forster City, CA). Fluorescence minus one controls (FMO) were used for each antibody combination to set the gates. Analysis was performed using the FlowJo software v10 (Ashland, OR).

Functional assays

The function of NK cells was assessed by measuring degranulation, through the degranulation marker CD107a [25], and IFN- γ production by intracellular staining. Isolated NK cells were cocultured with the MHC-I lacking target cell line K562 at a 1:1 effector:target ratio, along with CD107a-PE/Cy7 (clone H4A3, Biolegend) for 2–4 hours, adding the protein transport inhibitor GolgiStop (BD Biosciences) after the first hour. Cells were then 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. Pre-treatment versus post-treatment measurements were compared with an exact two-sided Wilcoxon signed-rank test. For each study (VOR and PNB), measurements from 5 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 pre-treatment, mid-treatment, and post-treatment. A Spearman r was calculated, but we also performed a Kendall Tau correlation, since there were several measures from each participant.

RESULTS

NK 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 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 an 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).

NK 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), while 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), while it remained unchanged after PNB treatment (Fig. 1C).

In vivo VOR treatment increases NK cell capacity to degranulate and produce IFN- γ

NK cell degranulation and IFN- γ production potential were evaluated after co-culture 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 ADCC 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).

CD16 and NKp46 are upregulated after VOR treatment

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 upregulated to some extent in most participants (Fig. 3A). Specifically, CD16 upregulation occurred exclusively within the CD56^{dim} subset, while 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 timepoints 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 $r=-0.51$, $p=0.0017$; 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 $r=-0.43$, $p=0.01$; Kendall's Tau: -3.8 (95% CI: -0.72 , 0.17), as well as 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 $r=0.35$, $p=0.041$; 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 current strategies aimed at eradicating persistent HIV infection consists of combining latency reversing agents 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 HDAC inhibitors on NK cell function. We have used samples from a clinical trial involving VOR (NCT01319383), in which 5 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 HDAC inhibitors. 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 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 and the frequency of NK cells, as well as with NK cell expression of CD16.

The question of whether HDACi suppress immune function is still under debate. Concern initially raised when Jones *et al.* reported that HDACis impaired CTL-mediated IFN- γ production and the elimination of HIV-infected cells *in vitro* [19]. 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 latency reversing agents 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, while Ogbomo *et al.* [28] and Pfeiffer *et al.* [29] observed a decrease in NK cytotoxicity. One potential explanation for these discrepancies are the different concentration and time of exposure used. In our experiments, as well as in Pace's, a concentration of 335 nM was used over 24 hours, to model peak VOR exposures *in vivo*, as VOR is cleared in less than 6 hours [30]. On the contrary, Ogbomo *et al.* used a continuous VOR concentration of 0.5–1 μ M over 96 hours, and Pfeiffer *et al.* 1 μ M over 48 hours. Regarding PNB, in our *ex vivo* study, we observed that a PNB exposure consisting of 20 ng/mL during 24 hours - which aimed to mimic physiologic conditions- increased NK cell death and reduced immune function [23], while 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 Panobinostat in HIV-1 infected individuals, Olesen *et al.* [31] found a correlation between HIV reservoir decline during Panobinostat 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, since IUPM data was 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, as well as 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 HDAC inhibitors do 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 , as well as 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.

ACKNOWLEDGEMENTS

We thank K. Sholtis, E. Stuelke and J. Kirchherr for technical assistance; J. Mathura and K. Mollan for statistical support; and J. Kuruc, C. Gay and C. Baker for clinical management. This work was supported by NIH U01-AI095052 and U19-AI096113 to DMM, and work in the UNC Flow Cytometry Core Facility is supported in part by the Center for AIDS Research award number P30-AI050410. We specially thank the participants in these clinical trials. Author contributions: CG conceived and designed the study; DMM and NSS provided input in study design and experiment planning; CG and BA performed the experiments; DMM, NMA, MT, TAR and OSS contributed reagents/materials; CG wrote the manuscript and NSS, DMM, NMA, MT and OSS reviewed it.

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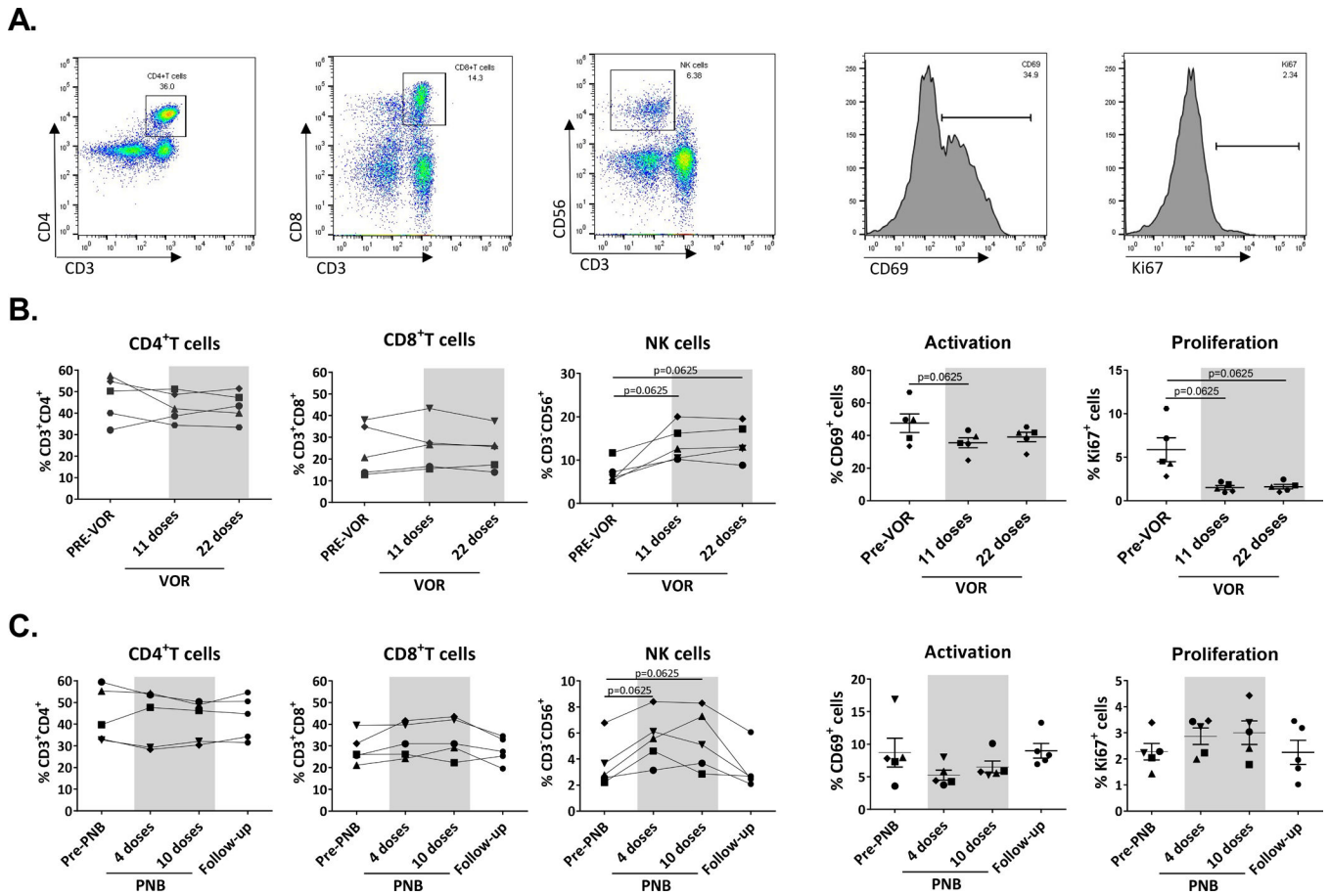


Fig. 1. Frequency of cell populations, proliferation and activation during HDAC inhibitor treatment.

PBMC 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.

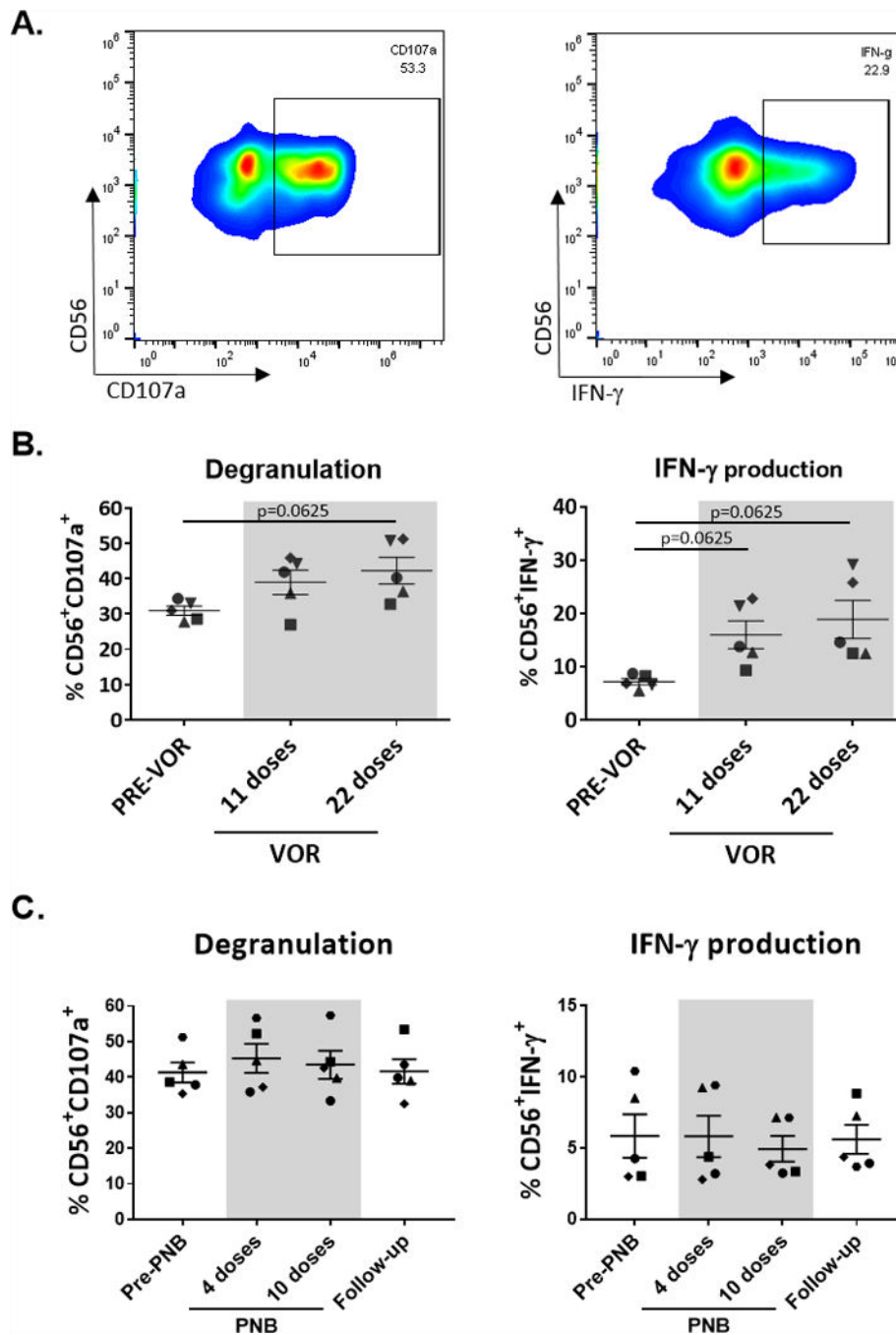


Fig. 2. NK cell cytotoxicity and cytokine production.

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.

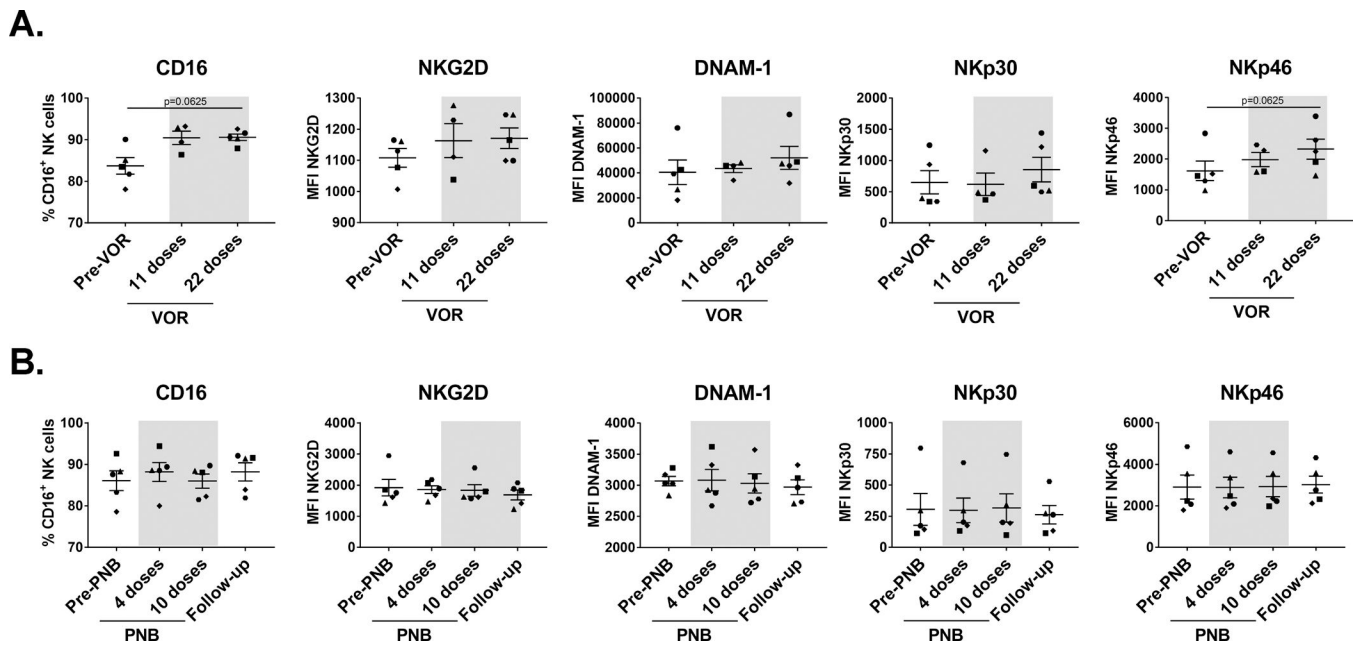


Fig. 3. Expression of activating receptors on NK cells.

NK cells were negatively isolated from thawed PBMC 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.

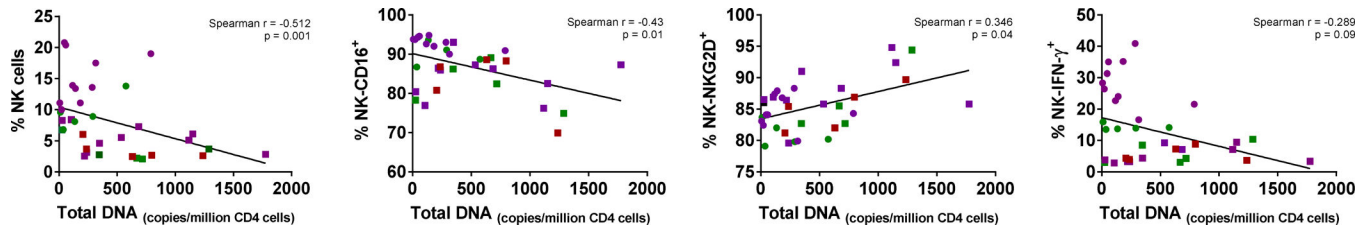


Fig. 4. Correlation with the HIV reservoir size.

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, while it correlated positively with NKG2D expression. Circles correspond to samples from the VOR study and squares to the PNB study. Green represent samples before HDACi treatment, purple during treatment and red after treatment.

Table 1.

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

| | Clinical Trial | # | Dose | Dosing | Reference |
|------------|-----------------------|----------|---------------------|---|----------------------------|
| | NCT01319383 | 8 | 400 mg | 1 dose | Archin, Nature 2012 |
| | NCT01319383 | 5 | 400 mg | 3 doses/week 8 weeks | Archin, JID 2014 |
| | NCT01365065 | 20 | 400 mg | 1 daily for 14 days | Elliott, PlosPath 2014 |
| VOR | | | | 1 dose | |
| | NCT01319383 | 16 | 400 mg | 1+1 after 48h 1+1 after 72h 10 doses every 72 hrs | Archin, JCI 2017 |
| PNB | NCT01680094 | 15 | 20 mg | 3 times/week every other week for 8 weeks | Rasmussen, Lancet HIV 2014 |
| RMD | NTC02092116 | 6 | 5 mg/m ² | 1 time/week for 3 weeks (intravenous) | Søgaard, PlosPath 2015 |

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