Innate Immune Activity Correlates with CD4 T Cell-Associated HIV-1 DNA Decline during Latency-Reversing Treatment with Panobinostat

Rikke Olesen,^a Selena Vigano,^{b,c} Thomas A. Rasmussen,^a Ole S. Søgaard,^a Zhengyu Ouyang,^b Maria Buzon,^{b,c} Arman Bashirova,^d Mary Carrington,^d Sarah Palmer,^e Christel R. Brinkmann,^a Xu G. Yu,^{b,c} Lars Østergaard,^a Martin Tolstrup,^a Mathias Lichterfeld^{b,c,f,g}

Infectious Disease Division, Aarhus University Hospital, Aarhus, Denmark^a; Ragon Institute of MGH, MIT, and Harvard, Cambridge, Massachusetts, USA^b; Harvard Medical School, Boston, Massachusetts, USA^c; Cancer and Inflammation Program, Laboratory of Experimental Immunology, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland, USA^d; Westmead Millennium Institute for Medical Research, University of Sydney, Sydney, Australia^e; Infectious Disease Division, Massachusetts General Hospital, Boston, Massachusetts, USA^f; Infectious Disease Division, Brigham and Women's Hospital, Boston, Massachusetts, USA^g

ABSTRACT

The pharmaceutical reactivation of dormant HIV-1 proviruses by histone deacetylase inhibitors (HDACi) represents a possible strategy to reduce the reservoir of HIV-1-infected cells in individuals treated with suppressive combination antiretroviral therapy (cART). However, the effects of such latency-reversing agents on the viral reservoir size are likely to be influenced by host immune responses. Here, we analyzed the immune factors associated with changes in proviral HIV-1 DNA levels during treatment with the potent HDACi panobinostat in a human clinical trial involving 15 cART-treated HIV-1-infected patients. We observed that the magnitude, breadth, and cytokine secretion profile of HIV-1-specific CD8 T cell responses were unrelated to changes in HIV-1 DNA levels in CD4 T cells during panobinostat treatment. In contrast, the proportions of CD3⁺ CD56⁺ total NK cells and CD16⁺ CD56^{dim} NK cells were inversely correlated with HIV-1 DNA levels throughout the study, and changes in HIV-1 DNA levels during panobinostat treatment were negatively associated with the corresponding changes in CD69⁺ NK cells. Decreasing levels of HIV-1 DNA during latency-reversing treatment were also related to the proportions of plasmacytoid dendritic cells, to distinct expression patterns of interferon-stimulated genes, and to the expression of the *IL28B* CC genotype. Together, these data suggest that innate immune activity can critically modulate the effects of latency-reversing agents on the viral reservoir and may represent a target for future immunotherapeutic interventions in HIV-1 eradication studies.

IMPORTANCE

Currently available antiretroviral drugs are highly effective in suppressing HIV-1 replication, but the virus persists, despite treatment, in a latent form that does not actively express HIV-1 gene products. One approach to eliminate these cells, colloquially termed the “shock-and-kill” strategy, focuses on the use of latency-reversing agents that induce active viral gene expression in latently infected cells, followed by immune-mediated killing. Panobinostat, a histone deacetylase inhibitor, demonstrated potent activities in reversing HIV-1 latency in a recent pilot clinical trial and reduced HIV-1 DNA levels in a subset of patients. Interestingly, we found that innate immune factors, such as natural killer cells, plasmacytoid dendritic cells, and the expression patterns of interferon-stimulated genes, were most closely linked to a decline in the HIV-1 DNA level during treatment with panobinostat. These data suggest that innate immune activity may play an important role in reducing the residual reservoir of HIV-1-infected cells.

Although for a long time regarded as an elusive goal, the development of clinical interventions that lead to a long-term, drug-free remission of HIV-1 infection is increasingly being recognized as a more and more realistic objective (1–4). This is in part related to the identification of patients with a sterilizing or functional cure of HIV-1 infection, who provide living evidence that, at least in principle, viral eradication or a drug-free remission of HIV-1 infection is possible (5, 6).

Latently infected CD4 T cells, in which a transcriptionally silent, replication-competent, but antiretroviral treatment-unresponsive form of HIV-1 can persist long term, are regarded as the predominant barrier against a cure for HIV-1 infection and represent the main reason for HIV-1 persistence, despite combination antiretroviral therapy (cART) (7, 8). The pharmacological induction of HIV-1 transcription in latently infected cells may render these cells susceptible to immune-mediated clearance and arguably represents one of the most promising and most broadly applicable strategies to target latently HIV-1-infected cells. Recently, results from pilot clinical trials evaluating the effects of

histone deacetylase inhibitors (HDACi) as latency-reversing agents have become available (9–12) and demonstrate that these agents are effective in increasing CD4 T cell-associated HIV-1

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Address correspondence to Mathias Lichterfeld, mlichterfeld@mgh.harvard.edu. R.O. and S.V. share first authorship, and M.T. and M.L. share last authorship.

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transcription *in vivo* in cART-treated HIV-1-infected patients. At least in the case of the HDACi panobinostat and romidepsin, this was associated with transient elevations of HIV-1 plasma RNA levels. However, induction of HIV-1 gene transcription by HDACi failed to translate into significant reductions in the size of the HIV-1 reservoir in most patients. Since latently infected CD4⁺ T cells can survive despite the effective pharmacological reactivation of HIV-1 gene transcription *in vitro* (13), it is possible that the reversal of viral latency by itself is in many cases insufficient to eliminate these cells and that additional immune-mediated effects are necessary to reduce the viral reservoir. However, the types of immune responses that are the most effective in eliminating cells with pharmacologically induced viral gene expression are unknown at present.

Previous studies have shown that HIV-1-specific CD8 T cells, which exert antiviral immune pressure through major histocompatibility complex class I-restricted cytotoxicity (14) and seem to influence set point viremia and spontaneous HIV-1 disease outcomes during untreated infection (15–17), can kill latently infected cells in which active HIV-1 transcription has been induced (13). However, in many cART-treated patients, these cells appear to be dysfunctional and insufficiently potent. Moreover, the immune effects of HIV-1-specific CD8 T cells are likely to be weakened by mutational escape in targeted epitopes (18, 19) and by possible inhibition through the intrinsic pharmacological effects of HDACi (20). Innate effector cell responses, preferentially mediated by natural killer (NK) cells and plasmacytoid dendritic cells (pDCs), may also have a role in restricting HIV-1 replication, as suggested by functional assays (21, 22), correlative cohorts studies (23, 24), immunogenetic associations (25), and *in vivo* experiments in animal models (26). However, the specific role of these cells in the context of pharmacological latency-reversing treatment remains unknown and represents an understudied area of investigation.

Although not significantly affecting HIV-1 DNA levels on a population level, reversal of viral latency with the HDACi panobinostat in a recent pilot clinical trial (12) translated into a wide spectrum of changes in viral DNA levels, suggesting that the effects of panobinostat on the HIV-1 reservoir are modulated by host factors. Interestingly, changes in HIV-1 DNA levels during panobinostat treatment were inversely correlated with viral rebound kinetics during an analytical treatment interruption (ATI), while baseline HIV-1 DNA levels were not. Moreover, a subgroup of four patients displayed pronounced decreases in HIV-1 DNA levels during panobinostat treatment, reaching approximately 70 to 80% of the baseline levels, and the time until viral rebound during ATI was the longest in these individuals. Together, these findings suggest that panobinostat-induced changes in HIV-1 DNA levels can translate into clinically significant effects. Here, we performed a detailed analysis of the innate and adaptive immune effects that were associated with decreases in the proviral DNA reservoir during treatment with the HDACi panobinostat in a human clinical trial. Remarkably and unexpectedly, we observed that innate effector cell responses and not HIV-1-specific CD8 T cells emerged as the major immune correlate of the decrease in HIV-1 DNA levels during panobinostat treatment.

MATERIALS AND METHODS

Study participants. Between September 2012 and February 2014 we conducted an investigator-initiated, single-arm, phase I/II clinical trial at Aar-

hus University Hospital, Aarhus, Denmark, to evaluate the ability of panobinostat to reactivate HIV-1 from latency. Details of the study have been published elsewhere (ClinicalTrials.gov registration no. NCT01680094) (12). Briefly, 15 HIV-1-infected adults on antiretroviral therapy (ART) with virological suppression for at least 2 years (plasma HIV-1 RNA load, <50 copies/ml) and CD4⁺ T cell counts above 500/μl received oral panobinostat at 20 mg 3 times per week every other week for 8 weeks, i.e., 4 cycles of treatment, while maintaining combination ART. The clinical and demographic characteristics of the study cohort were described previously (12).

Ethics statement. Ethics approval was obtained by the Ethics Committee at the University of Aarhus in accordance with the principles of the Declaration of Helsinki. Each patient provided written informed consent prior to any study procedures. The use of peripheral blood mononuclear cell (PBMC) samples from the Danish study patients for immunological and virological studies at Massachusetts General Hospital in Boston, MA, was approved by the Partners Human Research Committee.

Isolation and cryopreservation of PBMCs. Fresh blood was collected in heparin Vacutainer tubes (Becton Dickinson), and PBMCs were extracted according to the standard protocols. PBMCs were frozen in RPMI 1640 medium containing 40% heat-inactivated fetal bovine serum and 10% dimethyl sulfoxide and stored in liquid nitrogen.

Synthetic peptides. HIV-1 peptides corresponding to previously described optimal epitopes (27) were synthesized at the Massachusetts General Hospital Peptide Core Facility on an automated peptide synthesizer using the 9-fluorenylmethoxy carbonyl technology. Overlapping HIV-1 peptides spanning HIV-1 *gag* were obtained from JPT Innovative Peptide Solutions [PepMix HIV(GAG)].

ELISpot assays. Enzyme-linked immunosorbent spot (ELISpot) assays were carried out as described previously (28). Briefly, cryopreserved PBMCs were thawed and plated in 96-well polyvinylidene plates that had been precoated with 0.5 μg/ml of an anti-human gamma interferon (IFN-γ) monoclonal antibody (Mabtech). PBMCs were added at a concentration of 100,000 cells per well in a volume of 100 μl of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), HEPES buffer (10 mmol/liter), L-glutamine (2 mmol/liter), and penicillin-streptomycin (50 U/ml). Synthetic peptides corresponding to optimal HIV-1 epitopes were added to a single well at a final concentration of 2 μg/ml. The plates were incubated overnight at 37°C in 5% CO₂ and developed on the next day as described elsewhere (28). Wells containing PBMCs and medium with phytohemagglutinin or without any peptide were used as negative or positive controls, respectively, and run in triplicate on each plate. To calculate the number of specific T cells, the number of spots in the negative control wells was subtracted from the number of spots counted in each well.

Immune phenotyping by flow cytometry. Cryopreserved PBMCs were thawed and initially stained with blue viability dye (Invitrogen) for 20 min at 4°C. Afterwards, the cells were incubated at 4°C for 20 min with different combinations of appropriately titrated antibodies directed to the following surface markers: CD16 (clone 3G8), CD19 (clone HIB19), CD3 (clone HIT3a), CD11c (clone 3.9), HLA-DR (clone L243 or TU36), CD4 (clone RPA-T4), CD56 (clone HCD56), CD57 (clone HCD57), CD69 (clone FN50), NKG2A (clone Z199), CD14 (clone HCD14), CD86 (clone IT 2.2), CD3 (clone UCHT1), CD80 (clone L307.4), CD83 (clone HB15e), CD40 (clone 53C), CD123 (clone 7G3), CD27 (clone L128), and CD38 (clone HIT2). When necessary, the cells were preincubated for 10 min with 2 μl of FcR blocking antibodies. Afterwards, the cells were fixed in 2% paraformaldehyde solution, acquired on a five-laser Fortessa flow cytometer (Becton Dickinson), and analyzed using FlowJo X software (Tree Star). Analysis and presentation of cell distributions were performed using GraphPad Prism (version 6) and SPICE (version 5.32; <http://exon.niaid.nih.gov/spice>) (29) software. The number of lymphocyte-gated events ranged from 0.6×10^6 to 1×10^6 in these experiments.

Intracellular cytokine staining. In 14 of 15 patients, cryopreserved PBMCs were available for analysis of HIV-1-specific T cells by intracellu-

lar cytokine staining. PBMCs were thawed and rested for 18 h at 37°C in 5% CO₂. PBMCs were stimulated for 6 h at 37°C in 5% CO₂ with an HIV-1 Gag peptide pool (mix of 150 overlapping 15-mer peptides; final concentration, 2 µg/ml per peptide) in the presence of secretion inhibitors (GolgiStop at 0.7 µl/ml and Golgiplug at 1 µl/ml; Becton Dickinson) and antibodies against costimulatory molecules (anti-CD28 and anti-CD49d at 1 µg/ml each; Becton Dickinson). An unstimulated negative control and a positive control (staphylococcal enterotoxin B [SEB], 1 µg/ml; Sigma-Aldrich) were included for each time point. After stimulation, the cells were stained with viability dye (near-infrared amino-reactive dye; Invitrogen), followed by surface staining with antibodies against CD4 (clone OKT4; BioLegend), CCR7 (clone G043H7; BioLegend), CD8 (clone RPA-T8; Becton Dickinson), and CD45RA (clone HI100; Becton Dickinson). After fixation and permeabilization, intracellular cytokine staining was performed with antibodies against IFN-γ (clone B27; BioLegend), interleukin-2 (IL-2; clone MQ1-17H12; BioLegend), and tumor necrosis factor alpha (TNF-α; clone Mab11; BioLegend). A total of 800,000 events were collected per sample. Samples were analyzed on a Becton Dickinson FACSVerse cytometer, and data were analyzed using FlowJo (version 10.0.7) software. After defining gates for IFN-γ-, TNF-α-, and IL-2-positive events, we utilized Boolean combination gate analyses to create the full array of possible combinations (7 response patterns for 3 functions). Fluorescence-minus-one (FMO) controls were performed for surface marker CCR7 and the intracellular markers IFN-γ, IL-2, and TNF-α. On the basis of the background responses in unstimulated control samples, a positive HIV-1-specific response for CD8 and CD4 T cells was defined as having a background-corrected value greater than 0.06% or 0.02%, respectively. For the analyses of the median fluorescence levels of signals corresponding to selected cytokines, only samples from patients with a positive response to HIV-1 antigens were included.

Genotyping assays. HLA class I and KIR typing was performed by sequence-specific PCRs as described earlier (30). *IL28B* alleles were genotyped according to a protocol described elsewhere (31).

Gene expression profiling by microarrays. Isolated PBMCs were lysed in PAXgene blood RNA tubes as recommended by the manufacturer (PreAnalytiX, Switzerland) and stored at -80°C. Subsequently, RNA was extracted using a PAXgene blood RNA kit (Qiagen) and subjected to whole-genome transcriptional profiling using Affymetrix human transcriptome array (version 2.0) microarrays according to standard protocols. Data retrieved from the Affymetrix platform were background corrected and quantile normalized by the Affymetrix array studio program. A total of 67,528 transcripts were used to calculate the log₂ fold change in gene expression intensity between the beginning and the end of panobinostat therapy. Using Pearson's correlation, these changes were correlated to the corresponding changes in CD4 T cell associated HIV-1 DNA levels. Transcripts associated with changes in HIV-1 DNA levels were analyzed using Ingenuity pathway analysis (IPA).

Statistics. Data are expressed as individual data plots or as the median or mean, as indicated below. Bivariate comparisons between different time points were performed using Wilcoxon matched-pairs signed-rank tests. Spearman correlations were used for correlations at single time points. Generalized estimated equations (GEEs) were used to compute correlations across multiple time points. Statistical analyses and illustrations were performed using GraphPad Prism (versions 6 and R) software.

RESULTS

HIV-1-specific CD8 T cells are unrelated to HIV-1 DNA levels during HDACi therapy. To investigate the immune parameters associated with changes in HIV-1 DNA levels during treatment with panobinostat, we initially focused on the analysis of HIV-1-specific T cells. Using longitudinally collected PBMC samples from cART-treated HIV-1-infected patients undergoing four 1-week cycles of oral panobinostat treatment (12) (see Fig. S1 in the supplemental material), we performed sensitive IFN-γ ELISpot assays to measure the frequency of IFN-γ-secreting HIV-

1-specific CD8 T cells after stimulation with peptides corresponding to optimal CD8 T cell epitopes matched to the patients' individual HLA class I background. These studies indicated that the total magnitude and breadth (i.e., the number of targeted epitopes) of CD8 T cell responses recognizing optimal HIV-1 CD8 T cell epitopes mildly increased during treatment with panobinostat (Fig. 1A and B).

We subsequently used polychromatic flow cytometry to analyze the intracellular secretion of IFN-γ, IL-2, and TNF-α in HIV-1-specific T cells after stimulation with a library of overlapping peptides spanning HIV-1 *gag* (see Fig. S2 in the supplemental material). Overall, we found that treatment with panobinostat led to a slight and transient reduction in the relative proportion of total naive and central memory (CM) CD8 cells, while the proportions of effector memory (EM) and terminally differentiated (TD) CD8 T cells reciprocally increased (Fig. 1C). Cytokine-secreting HIV-1-specific CD8 T cells consisted mostly of IFN-γ-monoproducing and IFN-γ/TNF-α-coproducing EM and TD cells. The relative proportions of EM, TD, as well as unfractionated cytokine-secreting HIV-1-specific CD8 T cells during panobinostat treatment were unchanged relative to the baseline levels (Fig. 1D and E; see also Fig. S3 in the supplemental material). In addition, we observed no significant changes in the median fluorescence intensities of signals corresponding to IFN-γ or TNF-α production during or after panobinostat treatment compared to those at the baseline (see Fig. S4A to F in the supplemental material). Cytokine-secreting CD8 T cells specific for the staphylococcal enterotoxin B (SEB) antigen were also largely unaffected by treatment with panobinostat (see Fig. S5A to H in the supplemental material).

To gain insight into the possible effects of HIV-1-specific T cells against the viral reservoir during treatment with panobinostat, we correlated the HIV-1-specific T cell responses to the corresponding levels of CD4 T cell-associated HIV-1 DNA, as CD4 T cells are the reservoir biomarker associated with the kinetics of viral rebound during analytical treatment interruptions in our clinical study (12) (see Fig. S1B in the supplemental material) and other clinical studies (32, 33). The magnitude and breadth of HIV-1-specific CD8 T cells determined by ELISpot assays and the proportions of HIV-1-specific CD8 T cells secreting any combination of the analyzed cytokines at the baseline and throughout the study were statistically unrelated to the baseline levels of CD4 T cell-associated HIV-1 DNA or to the relative changes in HIV-1 DNA levels during panobinostat treatment (Fig. 1F to I; see also Fig. S3 and S6 in the supplemental material). The HIV-1-specific CD8 T cell responses and the changes in HIV-1 DNA levels in CD4 T cells during panobinostat treatment did not significantly differ between carriers of HLA class I alleles associated with improved outcomes of untreated HIV-1 infection (i.e., *HLA-B57* and *-B27*) and other patients (see Table S1 in the supplemental material). Moreover, the patients with a more pronounced decline in HIV-1 DNA levels during panobinostat treatment (see Fig. S1C in the supplemental material) did not differ from the remaining study cohort in terms of the magnitude, breadth, or the cytokine secretion profile of HIV-1-specific CD8 T cells (data not shown).

Baseline levels of polyfunctional HIV-specific CD4 T cells are predictive of HIV-1 DNA decline during panobinostat treatment. In analogy to the findings in the CD8 T cell compartment, the relative proportions of naive CD4 T cells transiently declined during treatment with panobinostat, at the expense of a slight expansion of central memory, effector memory, and terminally

FIG 1 Evolution of HIV-1-specific CD8 T cells during latency-reversing treatment with panobinostat. (A and B) Magnitude and breadth of IFN- γ -producing HIV-1-specific CD8 T cells measured by ELISpot assays at the baseline and after panobinostat treatment. Horizontal bars show median values with interquartile ranges. (C) Evolution of the CD8 T cell subset composition during panobinostat treatment. Stacked bars represent means and SEMs. See Fig. S1 in the supplemental material for definition of the time points for sample collection. *, $P < 0.05$; **, $P < 0.01$. (D and E) Proportions of HIV-1-specific EM (D) and TD (E) CD8 T cells producing IFN- γ only or coproducing IFN- γ and TNF- α at the indicated time points. Horizontal bars show median values with interquartile ranges. Statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank test. (F to I) Baseline magnitude and breadth of IFN- γ -producing HIV-1-specific CD8 T cells determined by ELISpot assays (F), baseline proportions of total CD8 T cell EM and TD memory cell populations (G), and baseline proportions of the indicated cytokine-secreting HIV-1-specific CD8 T cells (H and I) plotted against the corresponding changes in the CD4 T cell-associated HIV-1 DNA level during treatment with panobinostat. Spearman correlation coefficients (r values) are shown. Post, after panobinostat treatment; Base, baseline; SFU, number of spot-forming units; Mio, million.

FIG 2 Evolution of HIV-1-specific CD4 T cells during latency-reversing treatment with panobinostat. (A) Evolution of the subset composition of total CD4 T cells during panobinostat treatment. Stacked bars represent means and SEMs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B, C) Proportions of HIV-1-specific CM and EM CD4⁺ T cells producing IFN- γ ; TNF- α , and IL-2 individually (B) or coproducing all three cytokines (C). Horizontal bars show median values and interquartile ranges. See Fig. S1 in the supplemental material for definition of time points of sample collection. (D to F) Associations between baseline proportions of total memory CD4 T cells (D) or baseline proportions of cytokine-secreting HIV-1-specific CD4 T cells (E, F) and the corresponding changes in CD4 T cell-associated HIV-1 DNA levels during treatment with panobinostat. Spearman correlation coefficients are shown. Statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank test. Boldface P values indicate statistically significant differences.

differentiated CD4 T cells (Fig. 2A). The patients with a pronounced HIV-1 DNA decline did not differ from the remaining study cohort in the baseline levels of CD4 naive/memory subsets or in changes in the CD4 T cell subpopulations during and after treatment with panobinostat (data not shown). Cytokine-secreting HIV-1 (*gag*)-specific CD4 T cells in our study patients included central memory or effector memory cells producing TNF- α only, IFN- γ and TNF- α , TNF- α and IL-2, or IFN- γ , TNF- α , and IL-2, in line with the findings of previous studies (34). The relative proportions of all of these HIV-1-specific CD4 T cell populations remained stable during panobinostat treatment (Fig. 2B and C; see also Fig. S7A to C in the supplemental material). Moreover, the median fluorescence intensities of the signals corresponding to the cytokines produced by HIV-1-specific memory CD4 T cells did not change during treatment with panobinostat (see Fig. S4G to I and S7D to H in

the supplemental material). The proportions of cytokine-secreting SEB-specific CD4 T cells were also unaffected by panobinostat treatment (see Fig. S5I to L in the supplemental material).

Correlation analysis demonstrated that the baseline proportions of total memory CD4 T cells, as well as HIV-1-specific CD4 T cells secreting IL-2, IFN- γ , or all three tested cytokines, were inversely associated with subsequent changes in the CD4 T cell-associated HIV-1 DNA level during panobinostat treatment (Fig. 2D to F). The proportions of cytokine-secreting HIV-1-specific CD4 T cells were unrelated to the absolute levels or relative changes in the HIV-1 DNA levels throughout the remainder of the study (see Fig. S8 in the supplemental material) or to the proportions of cytokine-secreting HIV-1-specific CD8 T cells (data not shown).

B cell responses. In prior animal studies, B cell-mediated immune activity was critical for elimination of malignant cells dur-

FIG 3 NK cell responses are correlated with HIV-1 DNA levels during panobinostat treatment. (A) Representative flow cytometry dot plots indicating the proportions of total, CD56^{dim} CD16⁺, and CD56^{bright} CD16⁻ NK cell populations during treatment with panobinostat. (B) Longitudinal evolution of the indicated NK cell populations during treatment with panobinostat. Cumulative data from all available study subjects are shown for the indicated time points, as defined in Fig. S1 in the supplemental material. Horizontal bars reflect the median. (C) Spearman correlations between the proportions of the indicated NK cell populations and the corresponding CD4 T cell-associated HIV-1 DNA levels at each indicated time point. (D) Correlations between the proportions of the indicated total, CD56^{dim} CD16⁺, and CD56^{bright} CD16⁻ NK cell populations and the corresponding CD4 T cell-associated HIV-1 DNA levels. Data from all study patients and all available study time points were cumulatively analyzed using generalized estimated equations adjusted for repeated measures. Boldface *P* values indicate statistically significant differences.

ing treatment with HDACi (35). To determine whether B cells may influence changes in HIV-1 DNA levels during treatment with panobinostat, we analyzed the proportions of total CD3⁻ CD19⁺ B cells, CD19⁺ CD27⁺ CD38⁺ plasma cells, CD19⁺ CD27⁻ CD38⁺ germinal center-like B cells, CD19⁺ CD27⁺ CD38⁻ memory B cells, and CD19⁺ CD27⁻ CD38⁻ naive B cells in our study cohort before and after treatment with panobinostat. We observed that none of these cell populations changed substantially during the treatment course (see Fig. S9 in the supplemental material). In addition, the relative proportions of these B cell populations were unrelated to the levels of HIV-1 DNA, and changes in the frequencies of the B cell populations were not associated with the corresponding changes in HIV-1 DNA levels (data not shown).

NK cell frequencies are associated with an HIV-1 DNA decline during panobinostat treatment. In the absence of a signal suggesting an involvement of CD8 T cell responses in modulating

the effects of HDACi on HIV-1 DNA levels, we subsequently focused on NK cells, which can kill HIV-1-infected cells through innate immune mechanisms. We found that the proportions of CD3⁻ CD56⁺ NK cells slightly increased during the initial weeks of treatment with panobinostat, but the levels at the end of treatment were not different from those at the baseline (Fig. 3A/B). The transient increases in NK cell frequencies during early stages of panobinostat treatment were mostly related to an increase in the CD56^{dim} CD16⁺ subset of NK cells, which has increased cytotoxic properties, while CD56^{bright} CD16⁻ NK cells, a cell subset characterized by lower cytotoxic activities but stronger abilities to secrete antiviral and regulatory cytokines, declined slightly (Fig. 3B).

Interestingly, we observed that the relative proportions of CD3⁻ CD56⁺ total NK cells, as well as the proportions of the CD56^{dim} CD16⁺ NK cell subset, were inversely associated with the corresponding levels of CD4 T cell-associated HIV-1 DNA at most time points throughout the clinical trial (Fig. 3C). In addition,

FIG 4 Associations between CD57⁺, NKG2A⁺, and CD69⁺ NK cell subpopulations and HIV-1 DNA levels during panobinostat treatment. (A, B) Data reflect the correlations between the proportions of the total, CD56^{dim}CD16⁺, and CD56^{bright}CD16⁻ NK cell populations expressing CD57 (A) or NKG2A (B) and the corresponding CD4 T cell-associated HIV-1 DNA levels. Data from all study patients and all available study time points were cumulatively analyzed using generalized estimated equations adjusted for repeated measures. (C) Correlation between relative changes in the indicated CD69-expressing NK cell populations and the corresponding changes in HIV-1 DNA levels. Data reflect the fold change at weeks 6, 9, and 12 relative to the baseline levels. Cumulative data from all time points and all available study subjects were analyzed using generalized estimated equations adjusted for repeated measures. Boldface P values indicate statistically significant differences. n.s., not significant.

using a generalized estimated equation (GEE) analysis model, we found that the cumulatively compiled proportions of CD3⁻CD56⁺ total NK cells and CD56^{dim}CD16⁺ NK cells from all study time points were negatively associated with the corresponding levels of HIV-1 DNA in CD4 T cells. Associations between the proportions of CD56^{bright}CD16⁻ NK cells and HIV-1 DNA levels also showed a negative trend but were less pronounced than those for CD3⁻CD56⁺ total NK cells and CD56^{dim}CD16⁺ NK cells and did not reach statistical significance (Fig. 3D). Consistent with this

observation, we found that the proportion of cells expressing CD57, which denotes a functionally distinct subset of highly cytotoxic NK cells (36), inversely correlated with the corresponding HIV-1 DNA levels when expressed on total CD3⁻CD56⁺ or CD56^{dim}CD16⁺ NK cells but not on CD56^{bright}CD16⁻ NK cells (Fig. 4A). On the contrary, expression of the inhibitory NK cell marker NKG2A on CD3⁻CD56⁺ total NK cells and CD56^{dim}CD16⁺ NK cells was positively associated with the corresponding HIV-1 DNA levels, while NKG2A expression on CD56^{bright}

FIG 5 Changes in plasmacytoid dendritic cells are linked to decreases in HIV-1 DNA levels during treatment with panobinostat. (A) Longitudinal evolution of the proportions of indicated dendritic cell populations during treatment with panobinostat. Cumulative data from all available study subjects are shown for each indicated time point. Horizontal bars reflect the medians. (B) Spearman correlations between the relative changes in the proportions of plasmacytoid dendritic cells and the corresponding changes in HIV-1 DNA levels. Data reflect the fold change at weeks 6, 9, and 12 relative to the baseline levels. (C) Correlation between relative changes in the proportions of plasmacytoid dendritic cells and the corresponding changes in HIV-1 DNA levels. Cumulative data from all time points and all available study subjects were analyzed using rank-modified generalized estimated equations adjusted for repeated measures. Boldface P values indicate statistically significant differences.

CD16[−] NK cells was unrelated to the levels of HIV-1 DNA (Fig. 4B). Interestingly, the relative changes in HIV-1 DNA levels during panobinostat treatment were only weakly associated with the frequencies of any of the described NK cell populations but more strongly correlated with the corresponding changes in the proportion of CD3[−] CD56⁺ and CD56^{dim} CD16⁺ NK cells expressing the activation marker CD69, a C-type lectin known to trigger NK cell-mediated cytolytic activities (37); again, changes in the proportions of CD69⁺ CD56^{bright} CD16[−] NK cells were unrelated to the corresponding changes in HIV-1 DNA levels (Fig. 4C; see also Fig. S10 in the supplemental material).

We subsequently compared the NK cell responses between the four patients with a pronounced in HIV-1 DNA decline during panobinostat treatment and the remaining patients in whom HIV-1 DNA levels remained stable (see Fig. S1C in the supplemental material), using a linear regression model in a *post hoc* analysis. We found that total, CD56^{dim} CD16⁺, and CD56^{bright} CD16[−] NK cells were significantly more frequent in the four patients in whom decreases in HIV-1 DNA levels during panobinostat treatment were the most obvious (see Fig. S11 in the supple-

mental material). A similar observation was made for CD57[−] and CD69-expressing total and CD56^{dim} CD16⁺ NK cells (see Fig. S11 in the supplemental material). There was no significant association between the expression of KIR alleles and KIR/HLA class I combinations and changes in HIV-1 DNA levels during panobinostat treatment (data not shown; see Table S1 in the supplemental material).

Correlations between proportions of pDCs and HIV-1 DNA levels. The data presented above suggest a role for innate immune activity in influencing proviral DNA levels during latency-reversing treatment with panobinostat. We therefore next analyzed possible associations between dendritic cells (DCs) and the effects of panobinostat on the viral reservoir size. We observed that shortly after panobinostat treatment initiation in ART-treated HIV-1-infected patients, the relative proportions of total DCs transiently declined, but the levels tended to normalize during later stages of treatment and returned to nearly baseline levels after the completion of panobinostat treatment (Fig. 5A). These changes mostly occurred within the myeloid (conventional) DC (mDC) compartment, while the propor-

tions of pDCs, which can execute direct antiviral effector functions through the secretion of large amounts of IFN- α or direct cytotoxic effects, varied substantially among the individual study members but remained nearly stable on a cohort level (Fig. 5A). There was no association between the frequencies or relative changes in the amounts of total DCs or mDCs (compared to those at the baseline) and the corresponding levels of HIV-1 DNA during panobinostat treatment (data not shown). In contrast, changes in the relative proportions of pDCs during panobinostat treatment tended to inversely correlate to the corresponding changes in HIV-1 DNA levels (Fig. 5B). In addition, in a cumulative analysis of all longitudinal data from the study using a GEE model, we observed a strong inverse association between the relative changes in the proportions of pDCs and the corresponding changes in the levels of CD4 T cell-associated HIV-1 DNA (Fig. 5C). Moreover, the study patients with a pronounced HIV-1 DNA decline during panobinostat treatment (see Fig. S1C in the supplemental material) had significantly higher proportions of pDCs than the remaining study patients when stratified *post hoc* (see Fig. S12 in the supplemental material). The levels of expression of costimulatory molecules (CD80, CD86) and activation (HLA-DR) and maturation (CD83) markers on dendritic cells declined transiently during initial stages of treatment with panobinostat but returned to baseline levels later on; the absolute levels or relative changes in these parameters during panobinostat treatment were unrelated to the corresponding levels of HIV-1 DNA. Cytokine secretion of mDCs or pDCs was not assessed, as this cannot be reliably done using frozen cell samples.

Gene expression patterns associated with HIV-1 DNA decline. To further investigate the immune effects associated with a reduction of the viral reservoir during latency-reversing therapy, we used microarrays to analyze changes in gene expression patterns in PBMCs that correlated to the corresponding changes in HIV-1 DNA levels. Using linear regression analysis, we identified 764 transcripts with known functional annotations that were statistically significantly associated with changes in CD4 T cell-associated HIV-1 DNA levels during panobinostat treatment on the basis of a nominal *P* value of <0.05 (Fig. 6A). Functional pathways analysis demonstrated that these genes are involved in a wide spectrum of biological processes, including antigen presentation, regulation of inflammatory immune responses, cell growth and proliferation, as well as interactions with the complement system. Notably, transcripts associated with changes in CD4 T cell-associated HIV-1 DNA levels also included those for genes involved in the mTOR pathway and in the JAK/STAT kinase signaling pathway (Fig. 6A), both of which have previously been implicated in the regulation of the HIV-1 reservoir size and are actively being targeted by pharmaceutical interventions in ongoing HIV-1 eradication studies (AIDS Clinical Trials Group studies A5336 and A5337). The two functional pathways most strongly represented among all transcripts associated with changes in HIV-1 DNA levels during panobinostat treatment included those for the group of interferon-stimulated genes (ISGs) and those for genes involved in cell death and apoptosis. Indeed, a total of 257 ISGs listed in public databases (38) (see Table S2 in the supplemental material) were statistically significantly linked to changes in HIV-1 DNA levels (Fig. 6A and B; see Table S2 in the supplemental material); these genes can influence innate and adaptive immunity through a variety of mechanisms that focus on the modulation of che-

motaxis, lymphocyte homing and migration, or innate antiviral effector functions. Transcripts related to cell death and survival included 360 genes, 85 of which also belonged to the group of ISGs (Fig. 6A and C; see also Table S3 in the supplemental material). Since the expression patterns of ISGs and cell death genes can depend on the genotype at the *IL28B* locus (39–41), we analyzed the *IL28B* polymorphism in our study cohort. We observed that decreases in HIV-1 DNA levels during treatment with panobinostat were more pronounced in carriers of the *IL28B* CC genotype (Fig. 6D), which is associated with improved responses to treatment with recombinant IFN- α in hepatitis C virus infection (42) and has been linked to more functional innate, type I IFN-mediated antiviral immune activity (43). Together, these data support the hypothesis that innate, interferon-dependent immune activity may be relevant for reducing the viral reservoir during pharmacological disruption of HIV-1 latency.

DISCUSSION

Recent clinical studies indicate that HDACi are effective in reversing HIV-1 latency *in vivo*; however, successful pharmacological reactivation of viral gene expression failed to translate into significant changes to the HIV-1 reservoir in the majority of patients (10, 12). This observation likely corresponds to the findings of *in vitro* studies showing a remarkable ability of latently infected CD4 T cells to survive and proliferate at a time when active HIV-1 gene expression is pharmacologically induced (13). Together, these findings strongly suggest that the successful elimination of HIV-1-infected cells during latency-reversing treatment depends on host immune responses that kill cells in which viral gene expression is reactivated. Interestingly, experience from preclinical and clinical studies with HDACi in the context of oncologic diseases similarly indicates that elimination of malignant cells during HDACi therapy critically depends on effective host immune activity (35) and that combinations of HDACi and immune-based interventions may lead to more potent therapeutic effects against cancer cells (45–47). In the present work, we conducted a detailed and comprehensive analysis of innate and adaptive host immune responses during treatment with the HDACi panobinostat in ART-treated HIV-1-infected patients. Remarkably, these investigations revealed multiple complementary pieces of evidence suggesting that innate immune effects, rather than cytotoxic T cell responses, represent a distinguishing feature of patients with an HIV-1 DNA decline during panobinostat treatment and appeared to be most strongly associated with a reduction in the levels of HIV-1-infected cells during the pharmacological disruption of viral latency.

HIV-1-specific CD8 T cells are widely regarded as one of the most effective components of antiviral immune activity in humans, have a predominant influence on HIV-1 sequence evolution during untreated HIV-1 infection (48), and seem to represent the major correlate of HIV-1 immune protection in rare HIV-1 patients with spontaneous control of HIV-1 replication, who may serve as models for a functional cure of the disease (49). For these reasons, HIV-1-specific CD8 T cells have been considered promising immune factors for supporting the elimination of HIV-1-infected cells during treatment with latency-reversing agents, and at least two clinical trials evaluating combinations of HDACi with therapeutic vaccines aimed at boosting HIV-1-specific CD8 T cells are currently either in progress (ClinicalTrials.gov registration

FIG 6 A decrease in HIV-1 DNA levels during disruption of HIV-1 latency is associated with distinct patterns of ISG expression. (A) Identification of gene transcripts associated with changes in HIV-1 DNA levels during treatment with panobinostat. Differences in gene expression intensity at the end of the study (V12) compared to the gene expression at the baseline (V2) were correlated with the corresponding differences in HIV-1 DNA levels, using linear regression. The heat map reflects all transcripts that have a known functional annotation and are associated with the HIV-1 DNA level on the basis of a nominal *P* value of <0.05; subjects were ranked according to the change in HIV-1 DNA levels. EIF2, eukaryotic initiation factor 2 signaling; Gai, G protein alpha-dependent signaling. (B) Identification of ISGs associated with changes in HIV-1 DNA levels. Using linear regression, changes in the gene expression intensity of host transcripts defined as ISGs in public databases (www.interferome.org) were correlated to the corresponding changes in HIV-1 DNA levels. The heat map reflects ISGs associated with HIV-1 DNA on the basis of a nominal *P* value of <0.05; subjects were ranked according to the change in HIV-1 DNA levels. (C) Heat map reflecting all transcripts related to cell death and apoptosis that were statistically significantly associated with changes in HIV-1 DNA levels in CD4 T cells during panobinostat treatment on the basis of a nominal *P* value of <0.05; subjects were ranked according to the change in HIV-1 DNA levels. (D) Fold change in HIV-1 DNA levels between the beginning and end of treatment with panobinostat in patients stratified according to their *IL28B* CC and CT genotype. FC, fold change; pt, patient.

no. NCT02092116) or in advanced stages of planning (ClinicalTrials.gov registration no. NCT02336074). In the studies presented here, we found that cytokine-secreting HIV-1-specific CD8 T cells are detectable in the vast majority of patients and that their magnitude and breadth were mildly increasing during panobinostat treatment when measured using sensitive ELISpot assays. This suggests that the possible inhibitory effects of panobinostat on HIV-1-specific CD8 T cells observed *in vitro* (20) are less evident *in vivo* during drug administration to humans. More surprisingly, we found no positive evidence for a significant influence of cytokine-secreting HIV-1-specific CD8 T cells on the size of the proviral reservoir. Also, HLA class I haplotypes had no apparent effect on the dynamics of HIV-1 DNA levels. Apart from the dysfunction of HIV-1-specific CD8 T cells that has been frequently reported in HIV-1-infected patients (50, 51), even when receiving suppressive cART (13, 52), it is most likely that mutational escape in viral epitopes targeted by immunodominant CD8 T cell populations is responsible for these findings. Recent studies indeed found an accumulation of escape mutations in the vast majority of dominant CD8 T cell epitopes in proviral DNA, at least when treatment was initiated during chronic HIV-1 infection (18). Interestingly, the levels of HIV-1-specific CD4 T cells at the study baseline were predictive of a decline in the level of HIV-1 DNA during panobinostat therapy, but the levels of such cells at any other study time points and the relative changes of these cells throughout the study appeared to be unrelated to the corresponding levels of HIV-1 DNA. Given the dual role of HIV-1-specific CD4 T cells as a preferential cellular target for HIV-1 infection (53) and as helper cells supporting adaptive and innate effector cell populations (26, 50), the biological significance of this observation is difficult to ascertain at present and requires further investigation.

A predominant observation in this work is that NK cells were associated with the dynamics of HIV-1 DNA during panobinostat treatment. Although some studies have suggested that NK cells have antiviral effects against HIV-1 during untreated HIV-1 infection (22, 24), their contribution to antiviral immune defense during natural infection and in elite controllers is typically considered less dominant and inferior to the effects of HIV-1-specific CD8 T cells (49, 54). Our work suggests that in the context of viral reactivation treatment, these cells may have more important functions for eliminating residual HIV-1-infected cells. Indeed, the proportions of total and CD56^{dim} CD16⁺ NK cells were inversely associated with HIV-1 DNA levels throughout the study, particularly when expressing higher levels of CD57, a marker denoting enhanced cytotoxic activities (36), and lower levels of the inhibitory NK cell marker NKG2A. We also noticed that the relative changes in the amounts of total and CD56^{dim} CD16⁺ NK cells expressing the activation marker CD69, which arguably represents the most functionally active NK cell subset (37), were inversely associated with changes in HIV-1 DNA levels. The latency reversal by HDACi may possibly lead to the upregulation of activating NK cell receptors on CD4 T cells (55, 56), which may selectively sensitize these cells to the cytotoxic effects of NK cells, conceivably in the context of a cellular stress response to induced viral RNA (57). Intriguingly, latency reversal with HDACi may lead to the downregulation of HLA class I molecules, which may further promote NK cell-mediated killing of CD4 T cells in which viral gene expression is reactivated. Notably, the expression levels of HLA class I molecules, determined by gene expression profiling in microarray ex-

periments, were also inversely associated with changes in HIV-1 DNA levels during panobinostat treatment in our study.

In addition to NK cells, we found that decreasing levels of HIV-1 DNA during treatment with panobinostat were associated with the frequency of plasmacytoid dendritic cells, although these associations had weaker levels of statistical significance. pDCs can exert antiviral immune effects directly through killing of HIV-1-infected cells (59) or indirectly through secretion of large amounts of IFN- α , which has an adjuvant effect on different immune effector cells, including monocytes, NK cells, and T cells (60). Moreover, decreases in HIV-1 DNA levels seemed to be associated with changes in the expression of a large panel of ISGs and were more pronounced in carriers of the *IL28B* CC genotype, which appears to be related to more functional innate immune activity, at least in the context of hepatitis C virus infection (43, 61). These data correspond well to those from previous human and animal studies demonstrating that the pharmaceutical manipulation of IFN- α can reduce the reservoir of HIV-1-infected cells (62–64), likely through the activation of immune effector cells and changes in ISG expression patterns. Remarkably, Toll-like receptor 7 agonists, which can induce the endogenous secretion of IFN- α by pDCs, also seem to be able to reactivate HIV-1 from latently infected cells *in vitro* (65) and may reduce the viral reservoir *in vivo* (66). Overall, these results suggest that innate type I IFN immune responses may act as important immune factors for eliminating HIV-1-infected cells during the pharmacological reversal of viral latency.

Progress in understanding host factors and immune mechanisms that can support the elimination of HIV-1-infected cells during treatment with latency-reversing agents will depend to a large extent on small exploratory clinical trials, although the results from such studies are inherently limited by small sample sizes, uncontrolled study designs, and restrictions regarding the functional immune factors that can be analyzed from a limited number of cryopreserved PBMC samples. Hence, conclusions from such studies must be drawn cautiously and may not be easily generalizable. Nevertheless, such studies represent one of the most informative approaches to identification of the type of immune responses most effective in reducing the HIV-1 reservoir during disruption of viral latency. The data presented here suggest that innate immune activity is critical for reducing HIV-1 reservoirs during latency-reversing treatment and raise the hypothesis that combinations of latency-reversing agents with pharmacological interventions that stimulate type I IFN responses and/or innate immune effector cells may translate into the improved clearance of the reservoir of HIV-1-infected cells. Testing of such hypotheses in interventional human clinical trials may be informative for future efforts aiming at HIV-1 eradication and cure.

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