Single Cell RNA-Sequencing-based Analysis of CD4+ T-Cell Subset-Specific Susceptibility to Transcriptional Modulation by HIV-1 Latency-Reversing Agents

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Abstract

Shock-and-kill is one of the conceptually most advanced strategy towards establishment of an HIV-1 cure. Treatment with latency-reversing agents (LRAs), including histone deacetylase inhibitors with chromatin-remodeling capabilities, combined with anti-retroviral therapy, reactivates HIV-1 transcription in vivo. However, LRA treatment fails to significantly reduce the HIV-1 reservoir in HIV-1-positive individuals, indicating that it is probably insufficient to eliminate latently infected cells. The global and T-cell subset-specific impact of individual LRAs on the transcriptome of CD4⁺ T-cells, the main HIV-1 reservoir containing cell type in vivo, remains understudied. Here, using single cell RNA-sequencing, we characterize LRA treatment-induced alterations of CD4⁺ T-cell subset composition and of subpopulation-specific transcriptomes, using Vorinostat and Panobinostat as two prototypic HDAC inhibitors. Ex vivo exposure of CD4⁺ T-cells from an aviremic HIV-1-positive individual to Panobinostat markedly reduced the percentage of TREG cells. Furthermore, it altered expression of a multitude of interferon-regulated genes, resulting in suppression of several well-characterized antiviral genes, and in enhancement of selected interferon-regulated genes with proviral activities. These changes were most pronounced in T_N, T_CM, T_TM and T_EM, and less pronounced in T_REG. Exposure to Vorinostat resulted in a comparably mild change of cellular transcriptomic profile, regarding both the number of deregulated genes and their fold change of expression. Nevertheless, selected interferon-regulated genes exhibited a subset-specific expression profile upon Vorinostat treatment. Finally, some genes were deregulated by both treatments in a subset-specific manner. We conclude that treatment by both individual HDAC inhibitors induces an overall proviral milieu in CD4⁺ T-cells subsets. While this proviral state might be favorable for efficient HIV-1 reactivation, we hypothesize that it may impede the instruction of activation of cellular and adaptive immunity required for effective killing of reactivated cells.
Introduction

The development and implementation of highly active antiretroviral therapy (HAART) is established as an effective tool for suppressing viremia and improving the quality and duration of life for individuals living with HIV/AIDS. However, though these patients may exhibit undetectable plasma levels of the virus during optimal treatment, latently infected cells persist and expand (Vanhamel et al., 2019). In addition, cessation of treatment ultimately results in a return to a viraemic state within weeks in the vast majority of HIV-1-positive individuals (Hurst et al., 2015).

The HIV-1 reservoir is maintained by long-lived, latently infected CD4+ T-cells and cells from the monocyte/macrophage lineage. Among resting CD4+ T-cells carrying latent HIV-1, TCM and TTM predominate, but latent HIV-1 also resides in TN and TEM, albeit to a lower percentage (Chomont et al., 2009). The viral reservoir displays a very long half-life and slow decay rate, with predicted 73 years of antiretroviral therapy that would be required for eradication (Finzi et al., 1999; Siliciano et al., 2003). A main mechanism of persistence of the viral reservoir during antiretroviral treatment is clonal expansion through homeostatic proliferation, as indicated by a vast majority of CD4+ T-cells in treated patients sharing identical HIV-1 genome sequence and integration site (Cohn et al., 2015; von Stockenstrom et al., 2015; Wagner et al., 2014) or TCR sequence (Cohn et al., 2018). Of recent interest as a potential cure strategy is the so-named shock-and-kill approach. It is based on the administration of latency-reversing agents (LRAs), including histone deacetylase (HDAC) inhibitors, combined with antiretroviral treatment, and is anticipated to reactivate viral transcription in these reservoirs and thus target virus-infected cells for eradication by immunological or pharmacological means (Kim et al., 2018; Margolis et al., 2016). Clinical trials addressing the ability of individual LRAs to purge latent HIV-1 in vivo demonstrated reactivation of latent HIV-1 but no detectable reduction of the viral reservoir (Archin et al., 2012; Rasmussen et al., 2014; Sogaard et al.,
It has been suggested that individual LRAs display distinct specificities and potencies with regards to different T-cell sub-populations (Grau-Exposito et al., 2019), resulting in suboptimal responses in some, but not other subsets. Furthermore, PHA-reactivated CD4+ T-cells have been reported to display differential expression of genes related to cellular antiviral immunity (Cohn et al., 2018). Here, we exposed CD4+ T-cells isolated from an aviremic HIV-1-positive individual to Vorinostat and Panobinostat, respectively, and investigated, in individual T-cell subsets and by single cell RNA-sequencing, LRA-specific global transcriptomic changes and modulation of expression of cellular genes implicated in cell-intrinsic innate immunity.

Methods & Materials

CD4+ T-Cells from an Aviremic HIV-1 Patient

EDTA blood was collected in the context of the German HIV-1 Seroconverter Study (Machnowska et al., 2019) with approval of the local ethics committee (Ethical review committee of Charité University Medicine Berlin, Germany, EA 2/105/05). All study patients sign an informed consent form prior to enrollment. CD4+ T-cells were isolated using the EasySep Direct Human CD4+ T Cell Isolation Kit (STEMCELL Technologies). Cells were cultured at 2 x 10^6/ml in RPMI 1640 containing 10% heat-inactivated fetal calf serum (Sigma-Aldrich), 1% penicillin-streptomycin (Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 1x MEM Non-essential Amino Acid Solution (Thermo Fisher Scientific) and 1mM Sodium Pyruvate (Thermo Fisher Scientific).

Reagents
IL-2 and PHA were purchased from Roche and Oxoid, respectively. Panobinostat was obtained from Cayman Chem. Vorinostat was obtained through the NIH AIDS Research & Reference Reagent Program.

**IL-2/PHA Stimulation and Treatment with Latency-Reversing Agents**

CD4^+^ T-cells were mock-treated or treated individually with Vorinostat (500 nM), Panobinostat (50 nM) or IL-2 (20 U/ml)/PHA (1 µg/ml) for 48 hours.

**Single cell RNA sequencing**

Single Cell RNA-Seq libraries were prepared with the 10x Genomics platform using the Chromium Next GEM Single Cell 3’ Reagent Kits v.3.1 following manufacturer’s instructions. Quality control of the libraries was performed with the KAPA Library Quantification Kit (KK4854, Roche) and Agilent TapeStation (5067-5584, Agilent). Libraries were sequenced on a HiSeq 4000 (Illumina) device using the following sequencing mode: read 1: 28 bp, read 2: 91-100 bp, Index i7: 8 bp. The libraries were sequenced to reach ~20 000 reads per cell.

**Data Presentation and Statistical Analysis**

*Cell clustering, cell subset type identification and differential expression analysis*

Analysis of the scRNA data was performed using R Studio v3.6 (R Core Team, 2017) and the Seurat v3.1.4 package (Butler et al., 2018). Filtered Cell Ranger (v 3.1.0) matrices were imported into R and analysed according to the guidelines set out by the Satija Lab website. The data were initially filtered to remove cells with fewer than 500 unique detected genes to ensure adequate sequencing depth, as well as cells with unusually high numbers of gene reads, which likely represent cell multiplets, and cells with more than 10% mitochondrial DNA reads, as
these represent dead cells. After filtration, a total of 22715 cells remained. The data was log-normalised and scaled using the LogNormalize and ScaleData functions. Principal component analysis and linear dimensional reduction analysis using a Uniform Manifold Approximation and Projection (UMAP) plot were performed, where cells were clustered by means of a Shared Nearest Neighbor (SNN) graph, based on a K-Nearest Neighbor graph. In general, a resolution parameter of 1.0 or higher was used to cluster cells in order to retrieve a maximum amount of clusters. Identities of clusters were determined using a graph-based clustering approach and the list of pre-determined cell marker genes. Integration of datasets was performed using Seurat’s in-built toolset for finding ‘anchors’ between datasets (FindIntegrationAnchors) via a canonical correspondence analysis (CCA). Testing for differential gene expression was performed using a Wilcoxon sum rank test with a Bonferroni correction. Only genes with a natural log fold-change of at least ±0.25 (1.28 or 0.77 fold change) and that were expressed in at least 10% of cells in either the mock treated or LRA-treated sample were included, where an adjusted p-value of lower than 0.05 was considered significant. As the analysis focussed on CD4+ T cells, any clusters identified as CD8+ cells, NK cells (CD3D−, NKG7hi, GNLYhi) or dead cells (where the most significantly differentially regulated genes were mitochondrial in origin) were manually removed.

Cell trajectory and GSEA analysis

Cell trajectory/Pseudotime analysis was performed using the Monocle v2.14.0 package (Trapnell et al., 2014) according to the guidelines on the Monocle website. Data from the created Seurat object was imported into Monocle as raw counts (UMI). For each cell subset, genes identified to be significantly differentially regulated based on the above-mentioned criteria were used as the ordering genes for the Pseudotime analysis. The minimum spanning tree was generated using the DDRTree algorithm and the cells arranged along the resulting Pseudotime trajectory. This was also performed using all genes differentially expressed
between treated and mock-treated cells across all cell subsets. As very few (<10) Treg cells in the Panobinostat treatment could be identified, these were omitted from the trajectory analysis. Genes defining the branchpoint observed in the trajectory analysis were determined using a branch expression analysis model (BEAM). Gene set enrichment analysis (GSEA) was performed on the same genes used for ordering in the trajectory analysis using the fGSEA v1.12.0 package for R (Korotkevich et al., 2019). The analysis was performed using 10000 permutations, with the REACTOME curated pathway dataset (Jassal et al., 2020) obtained from the MSigDB R package (msigdb) v7.0.1 used as the input pathway list. Of the full lists of differentially regulated genes for each cell subset and treatment, genes that represented interferon-regulated genes (IRGs) were identified by cross-referencing these lists with the Interferome 2.01 database (Rusinova et al., 2013). Differential expression testing was then performed as described.

Additional data visualisation

Venn diagrams depicting the intersection of genes differentially expressed amongst different cell types were generated using the venn R package v1.9 (Dusa, 2020), while those depicting the intersection in different LRA treatments were generated using the VennDiagram R package v1.6.20 (Chen, 2018). Heatmaps were generated using GraphPad Prism (Version 8.3.0). All other plots were generated using the ggplot2 R package (Wickham, 2016).
Results

In order to characterize LRA-specific effects and CD4+ T-cell subpopulation-specific susceptibilities to individual LRAs, we exposed purified CD4+ T-cells isolated from an aviremic, HIV-1 positive individual (Table S1) to Vorinostat, Panobinostat, IL-2/PHA or left them mock-treated for 48 hours, and subjected them to single cell RNA-sequencing. We chose to test Vorinostat and Panobinostat due to their demonstrated efficacy to reactivate HIV-1 mRNA expression in vivo (Archin et al., 2012; Rasmussen et al., 2014). The employed concentrations and duration of Vorinostat and Panobinostat treatment correspond to the documented ability of the drugs to reactivated HIV-1 in vitro (Rasmussen et al., 2013; Tsai et al., 2016). We included IL-2/PHA treatment as a reference that we expected to result in maximum and relatively unspecific activation of T-cells. Finally, we decided to restrict our study on CD4+ T-cells from an aviremic HIV-1-positive individual, as opposed to T-cells from a healthy individual for two reasons. First, this strategy allowed us to consider potential changes of the T-cell composition and transcriptome in the context of a preceding HIV-1 infection and long-term antiretroviral treatment (Wang et al., 2019). Second, we aim to correlate, at the single cell level, identified transcriptomic signatures to patterns and efficiencies of HIV-1 mRNA expression.

We used a graph-based clustering approach and T-cell subset-specific marker genes to assign cells into different subsets (Fig. 1A-C). The cells were grouped into naive CD4+ T-cells (TN; CD8A-, CD3D+, CCR7+, IL7Rhi), central memory CD4+ T-cells (TCM; CD8A-, CD3D+, CCR7+, S100A4int, IL7R+, SELLhi) (Golubovskaya and Wu, 2016; MacLeod et al., 2009), transitional memory T-cells (TTM; CD8A-, CD3D+, CCR7lo, IL7R+, S100A4hi, SELLlo, GZMA-, CD27+) (Mahnke et al., 2013), effector memory CD4+ T-cells (TEM; CD8A-, CD3D+, CCR7-, IL7R+, S100A4hi, CD27+, GZMA+) (Golubovskaya and Wu, 2016; Weatherly et al., 2015), CD4+ regulatory T-cells (TREG; CD8A-, CD3D+, FOXP3+, IL2RA+) (Golubovskaya and Wu,
2016) and a subset we defined as effector lo CD4+ T-cells (CD8A+, CD3D+, CCR7, IL7R+, S100A4hi, CD27+, GZMAlo), which defined a separate cluster expressing low levels of GZMA but relatively high levels of other effector molecules (GNLY, GZMB).

While exposure to Vorinostat did not grossly alter the CD4+ T-cell subtype composition, individual treatment with Panobinostat and IL-2/PHA substantially altered relative percentages of CD4+ T-cell subsets (Fig. 1B-C). Panobinostat treatment induced an almost entire eclipse of TREG cells (0.1%, versus 6% in mock-treated cells). Furthermore, it enlarged the pool of TN cells (38.4% versus 26.2% in mock-treated cells). IL-2/PHA treatment resulted in appearance of a separate cluster of cells we referred to as effector lo CD4+ TEM-cells. We hypothesize that this additional cell cluster represents a transitory state of differentiation between TTM and TEM-cells, which would be in line with the canonical role of IL-2 as an inducer of T-cell differentiation towards effector memory status (Ross and Cantrell, 2018) (Figure 1A-C).

LRA- and CD4+ T-cell subset specific modulation of the transcriptome
Integrated analysis of the composition of CD4+ T-cells in the absence and presence of a specific treatment revealed a noticeably altered topography of the UMAP in the case of treatment with Panobinostat and IL-2/PHA relative to Vorinostat. Indeed, though Seurat’s toolkit for integrated analysis is robust in promoting cell subset-specific clustering despite treatment-specific effects, we observed the emergence of several clusters comprising almost entirely of cells from the LRA treatment (TN cells in the case of Panobinostat, as well as TN, TCM and TTM cells in the case of IL2-PHA treatment). Though this observation alone is insufficient to make a solid conclusion about gene expression profiles, it is in agreement with our observations that the transcriptomes of Panobinostat and IL-2/PHA treated cells appear to be radically altered in comparison to the Vorinostat treatment (Fig. 2A).

The number of statistically significantly (p < 0.05, 0.78≤fold-change≥1.28) deregulated genes varied between individual treatments and among CD4+ T-cell subsets. Exposure to
Vorinostat induced deregulation of relatively few genes in all five subpopulations (Fig. 2B, Table S2). Panobinostat treatment resulted in altered expression of several hundred genes in most CD4+ T-cell subsets, except T_{REG} CD4+ T-cells, which presented a lower degree of responsiveness to this HDAC inhibitor treatment compared to the other subsets. IL-2/PHA treatment changed the transcriptome in all five sub-populations (Fig. 2B). In all CD4+ T-cell subsets except T_{REG}, most genes deregulated by Vorinostat were also modulated by the two other treatments (Fig. 2C).

The relatively few genes modulated by Vorinostat were evenly distributed among the CD4+ T-cell sub-populations, with T_{REG} presenting the highest number of unique differentially expressed genes (DEGs) (29 DEGs), and T_{TM} displaying not a single specific DEG (Fig. 2D). All individual T-cell subsets expressed a relatively unique set of 43-170 different genes in the context of treatment with Panobinostat, and presented some overlap between T_{N} and T_{CM} (85 DEGs), T_{TM} and T_{EM} (43 DEGs), and T_{TM} and T_{CM} (51 DEGs), indicating that treatment induces a T-cell subset-specific transcriptomic change (Fig. 2D). All subsets excluding T_{REG} shared 593 DEGs upon Panobinostat treatment. IL-2/PHA treatment induced a large number of DEGs in all subsets, 876 deregulated in all five sub-populations (Fig. 2D).

In order to investigate whether the altered gene expression profile in the Panobinostat treated sample induced a single or multiple cell trajectories and which pathways were most associated with this profile at cell subset level, we performed Pseudotime and gene set enrichment analysis (GSEA). Genes significantly differentially regulated in the treated versus mock cells for each subset were used as ordering and input genes, respectively. As there were very few T_{REG} cells in the Panobinostat treated sample, this cell subset was omitted from the analysis as we concluded that the results would likely not be conclusive (Fig. 2E). Cell trajectory analysis revealed that for all of the subsets, except T_{N} cells, the altered gene expression profile defines a single cell fate. For T_{N} cells, branched expression analysis modeling (BEAM) revealed that the differentially expressed genes defining the branchpoint are
comprised largely of genes encoding ribosomal structural proteins (Supplementary Figure S1).

Interestingly, the positively and negatively enriched pathways in all subsets were similar (Fig. 2F). Generally, positively enriched pathways related to either cell stress, as in the DNA repair, cellular response to stress and cellular senescence pathways, or to cell proliferation and productivity, as in the case of the cilium assembly, nonsense mediated decay (NMD), rRNA processing and organelle biogenesis and maintenance pathways (Han et al., 2018; Sanchez and Dynlacht, 2016). In contrast, the negatively enriched pathways relate largely to immune function, such as the TCR signaling, adaptive immune signaling, cytokine immune signaling and immunoregulatory interactions between a lymphoid and non-lymphoid cell, suggesting that Panobinostat-treated cells may exhibit an impaired immune response capability. This migration of cells towards a single cell fate defined by Panobinostat treatment was recapitulated in the Pseudotime analysis combining all CD4+ T-cell subsets. Interestingly, as the path through Pseudotime is followed, the root state (containing largely T_N cells) branches off into two cell fates – a branch containing entirely mock treated cells with a high representation of T_EM cells and a branch containing all of the Panobinostat treated cells. This branching implies that the trajectory of T_EM cells is largely contrarian to the transcriptomic profile induced by treatment, which is in line with the observation that Panobinostat appears to downregulate immune response pathways. Simultaneously, despite some cells having a very different transcriptomic profile to begin with, it becomes clear that Panobinostat induces a single and similar cell fate across all cell subsets.

LRA treatment alters the expression of IRGs in CD4+ T-cells in a treatment- and subset-specific manner

In order to better understand how LRA treatment modulates the landscape of interferon-regulated genes (IRGs), we determined the fraction of all DEGs representing IRGs according to and using Interferome v2.01 (Rusinova et al., 2013). Interestingly, the majority of DEGs
represented IRGs (80%, 68%, 46% for Vorinostat, Panobinostat and IL-2/PHA, respectively).

Consequently, due to the overall higher degree of gene expression alteration, treatment with IL-2/PHA and Panobinostat led to a much broader panel of differentially expressed IRGs than did treatment with Vorinostat (Fig. 3A). While Vorinostat treatment tended to result in a balanced number of up- and downregulated IRGs, Panobinostat treatment provoked more than twice the number of downregulated versus upregulated IRGs in TCM, TTM and TEM cells. IL-2/PHA, in contrast, consistently resulted in a considerably larger ratio (generally at least two- to three-fold) of upregulated, compared to downregulated, IRGs (Figure 3A).

While Vorinostat treatment resulted in 75 differentially regulated IRGs across all CD4+ T-cell subsets, individual exposure to Panobinostat and IL-2/PHA resulted in 1139 and 1342 differentially expressed IRGs, respectively. Of these, only 5, 29 and 509 for the Vorinostat, Panobinostat and IL-2/PHA exposure, respectively, were identified as differentially regulated across all T-cell sub-populations within the individual treatments (Fig. 3B), implying that alterations in the cellular IRG expression profile are predominantly subset-specific for all LRAs. Furthermore, the altered IRG profile observed in each cell subtype was largely determined by the specific LRA. Generally, while there was a sizeable overlap in IRGs differentially regulated by both Panobinostat and IL-2/PHA, we observed a substantial number of IRGs whose expression was only affected by treatment with either Panobinostat or IL-2/PHA. For example, in T_N cells, only 18 IRGs are differentially expressed upon all three individual treatments, while 412 IRGs were affected by Panobinostat and IL-2/PHA but Vorinostat and a further 363 and 891 genes were uniquely affected by Panobinostat and IL-2/PHA, respectively (Figure 3C).

In the context of Vorinostat and Panobinostat treatment, we focused our attention on significantly deregulated IRGs whose gene products have been characterized to serve as antiviral restriction factors, to be implicated in downstream signaling upon PRR triggering through viral PAMPs, and to be otherwise implicated in HIV-1 biology. Strikingly, analysis of
their expression level upon treatment (in comparison to mock treatment), revealed a marked
downregulation in most, but not all subsets. Importantly, some key anti-HIV-1 factors, such as
APOBEC3G (Sheehy et al., 2002), IFITM1-3 (Compton et al., 2014; Foster et al., 2016; Tartour
et al., 2014), MX2 (Goujon et al., 2013; Kane et al., 2013; Liu et al., 2013) and SAMHD1
(Hrecka et al., 2011; Laguette et al., 2011) were downregulated in the context of Panobinostat
treatment for the majority of cell subsets. GBP5, a gene encoding a cellular factor that interferes
with HIV-1 Env processing and maturation (Braun et al., 2019; Krapp et al., 2016) displayed a
subset-specific regulation of expression, with most pronounced downregulation in T_{REG}, weak
downregulation in T_N and T_{TM}, virtually no alteration in T_{CM} and upregulation in T_{EM}.

Interesting exceptions to this trend were CDC37 and HSP90A1, whose gene products act in
complex on regulation of HIV-1 latency (Anderson et al., 2014) and LGALS1, an IRG encoding
a protein facilitating HIV-1 attachment to target cells (Mercier et al., 2008; Ouellet et al., 2005;
St-Pierre et al., 2011). The latter and all three factors were markedly upregulated by Vorinostat
and Panobinostat, respectively, in most T-cell subsets (Fig. 3D-E). Importantly, some key
antiviral factors, such as TRIM56 (Kane et al., 2016 PMID 27631702), SUN2 (Donahue et al.,
2016 PMID: 26865710 SUN2) and IFITM1-3 (Tartour et al., 2014 PMID 25422070; Foster
et al., 2016; Compton et al., 2014 PMID:25464829) were downregulated by Panobinostat
treatment for the majority of cell subsets (Figure 3D-E). Taken together, these data imply that
Vorinostat and Panobinostat treatments may result in an abrogated innate immune response and
potentially a more proviral milieu in CD4+ T-cells.
Discussion

The eminent aim of a LRA treatment in the context of HIV-1 cure is reactivation of HIV-1 mRNA and protein expression. This is supposed to occur through relatively unspecific remodulation of cellular chromatin by currently tested LRAs, boosting transcriptional reactivation of latent HIV-1 genomes but concomitantly resulting in expression changes of a multitude of cellular genes. How those transcriptomic changes of cellular genes indirectly impact HIV-1 reactivation and susceptibility of cells to re-infection remains understudied. An optimal shock-and-kill regimen must be, on the one hand, proviral with regards to all post-integrational steps. It should support or even boost Tat-mediated trans-activation of transcription, protein expression, and cellular trafficking of viral proteins in order to turn previously latent cells in immunologically visible cells which display Env and MHC-presented viral epitopes at the surface. On the other hand, an optimal shock-and-kill strategy must also trigger antiviral immune mechanisms, including cell-mediated immunity by CD8- and NK-cell-mediated cytotoxicity. Along this line, it has been suggested that a reduction of HIV-1 DNA levels during latency reversal is associated with distinct patterns of ISG expression (Olesen et al., 2015). HDAC inhibitors have clearly demonstrated their ability to promote reactivation of HIV-1 mRNA synthesis and protein expression in vivo and in vitro (Archin et al., 2012; Rasmussen et al., 2013; Rasmussen et al., 2014; Sogaard et al., 2015; Tsai et al., 2016). In ex vivo-treated CD4+ T-cells, Vorinostat, Panobinostat and Romidepsin enhanced HIV-1 de novo infection (Lucera et al., 2014), raising concerns on potential spread of HIV-1 during shock-and-kill regime in body compartments insufficiently targeted by HAART. On the contrary, another report presented rather an antiviral and no effect of Romidepsin and Panobinostat, respectively (Jonsson et al., 2015). In addition to the missing or controversial knowledge on the net cellular state that is overall induced in LRA-treated CD4+ T-cell cultures, there is no information on subset-specific impact of individual LRAs, and also not if, within a given T-cell subset, there are individual susceptibilities to LRA treatment at the single cell level. Given the preferential
occurrence of latent HIV-1 in TCM and TTM, and potential subtle particularities of cells harboring latent HIV-1 DNA as opposed to their HIV-1 negative counterparts, the characterization of the susceptibility to LRA treatment at the greatest possible level of detail is urgently required.

Vorinostat induced rather subtle changes in the CD4+ T-cell subset composition and in the cellular transcriptome of individual T-cell subpopulations. A previous report using a higher concentration and shorter treatment duration suggested that genes promoting and inhibiting HIV-1 transcription were both upregulated in Vorinostat-treated CD4+ T-cells (Beliakova-Bethell et al., 2019). Based on the current status of our analyses, we observe that several IRGs with documented anti-HIV-1 activity are downregulated in most T-cell subsets, and one interferon-modulated gene with a proviral function (LGALS1) is upregulated. Based on these observations, Vorinostat seems to promote an overall rather proviral state. Interestingly, not all subtypes were equally susceptible to this pattern, pointing to the idea that TEM may be slightly more resistant to the Vorinostat-mediated suppression of antiviral IRGs. Future studies are required to identify if this correlates to a less efficient reactivation of latent HIV-1 in this particular CD4+ T-cell subtype. Interestingly, Vorinostat has been reported to reactivate endogenous retroviruses (White et al., 2018), and investigation of this issue in our dataset is imminent.

Exposure of CD4+ T-cells to Panobinostat for 48 hours resulted in an almost entire disappearance of TREG cells. This finding appears to contradict results obtained upon in vivo administration of Panobinostat to HIV-1 patients in the context clinical latency reversal trials, which demonstrated a profound and reversible expansion of TREG cells (Brinkmann et al., 2018). One possibility to reconcile those findings is that Panobinostat’s effect may not be restricted to CD4+ T-cells in vivo and may have triggered expansion of TREG via indirect effects, involving other cell types than CD4+ T-cells. Importantly, another study suggested that Panobinostat treatment in vitro, and in the context of co-administration with the cytidine analog azacitidine
in vivo, lowered the frequency of a specific T\textsubscript{REG} subset that is elevated in frequency in the peripheral blood of acute myeloid leukemia patients (Govindaraj et al., 2014).

Panobinostat treatment clearly modulated the transcriptional landscape of all CD4\textsuperscript{+} subsets. We currently cannot exclude that the apparently smaller pool of deregulated genes in T\textsubscript{REG} may be related to the reduction of frequency of this subset itself. Nevertheless, the other four T-cell subsets generally shared the downregulation of IRGs with antiviral activity, with some notable exceptions for individual genes in individual subsets. We are currently analyzing the T-cell subpopulation-specific expression pattern for HIV-1 latency modulating genes.

The preliminary findings of this study must be interpreted with caution. They are based on findings obtained from cells from a single donor, and currently performed analyses on cells from additional donors will be included very soon. Furthermore, the correlation analysis of the identified, partially subtype-specific cellular transcriptomic patterns to HIV-1 mRNA expression is an important ongoing task. Interestingly, preliminary experiments indicate that despite the limited number of cells analyzed, and despite the relative rarity of HIV-1-positive cells in the peripheral blood of aviremic individuals, this experimental pipeline allows the identification of reads that map to the HIV-1 genome.

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Author Contributions

J.K. and C.G. conceived and designed the experiments; J.K., J.J., S.N.V. and M.S. performed the experiments; J.K., D.P., E.W., C.F., K.M. and C.G. analyzed the data; J.K., D.P. and C.G. drafted the manuscript; J.K., D.P. and C.G. wrote the manuscript; S.S., N.B., M.L. and C.G. supervised the research.

Declaration of Interests

None
Legends

Figure 1. LRA treatment modulates CD4+ T-subset composition

(A) UMAP plots of mock-, Vorinostat-, Panobinostat- and IL-2/PHA-treated CD4+ T cells. The individual CD4+ T-cell subsets are indicated by colouration.

(B) Pie charts indicating the relative percentage of individual CD4+ T cell subset for indicated conditions.

(C) Raw counts and relative percentages of individual CD4+ T cell subsets for indicated conditions.

Figure 2. LRA- and CD4+ T-cell subset specific modulation of the transcriptome

(A) UMAP plots of the integrated analysis Vorinostat, Panobinostat and IL-2/PHA treated samples with the mock treated sample.

(B) Volcano plots of all significantly differentially regulated genes in each LRA treatment relative to the mock sample (minimum log e fold change of ±0.25, Bonferroni corrected p-value <0.05, Wilcoxon rank-sum test). Upregulated genes are indicated in red, downregulated genes in blue.

(C) Overlap in differentially regulated genes between treatments within each cell subset.

(D) Overlap of differentially regulated genes between cell subsets within each treatment.

(E) Pseudotime cell trajectory analysis and GSEA analysis using genes differentially regulated between the mock and Panobinostat treatments for T_N, T_CM, T_TM and T_EM cells, and for total CD4+ T-cells. Cells are represented as arrayed along the Pseudotime trajectory, coloured by both treatment and their pseudotime scores.

(F) The top five most significantly positively and negatively enriched pathways (based on the associated p-value) for each cell subset are indicated, along with the corresponding normalised enrichment score (NES).
Figure 3. LRA treatment alters the expression of IRGs in CD4+ T-cells in a treatment- and subset-specific manner

(A) Volcano plots of all significantly differentially regulated IRGs in each LRA treatment relative to the mock sample (minimum log e fold change of ±0.25, Bonferroni corrected p-value <0.05, Wilcoxon rank-sum test). Upregulated genes are indicated in red, downregulated genes in blue.

(B) Overlap of differentially regulated IRGs between cell subsets within each treatment.

(C) Overlap in differentially regulated IRGs between treatments within each cell subset.

(D) Heat maps indicating fold change in expression between mock and treated samples for selected IRGs with potential implications for anti-viral activity in the Vorinostat and Panobinostat treatments. The normalised fold change in IRG expression is shown (expression in mock treatment = 1). Crossed-out (X) fields represent genes that were not expressed in at least 10% of either the mock or treated sample. Average fold changes in gene expression for significantly differentially expressed IRGs are indicated above the corresponding violin plot for each cell type (Wilcoxon rank-sum test, Bonferroni corrected p-value <0.05).

(E) Violin plots showing normalised expression for selected IRGs with potential implications for anti-viral activity in the Vorinostat and Panobinostat treatments. Numbers indicate fold change expression of significantly deregulated IRGs.
References


cells with HIV integrated into cancer genes contributes to persistent infection. *Science* 345, 570-573.


Kazmierski et al., Figure 1

A

Mock  Vorinostat  Panobinostat  IL-2/PHA

B

C

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<th>Treatment</th>
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<th>Panobinostat</th>
<th>IL-2/PHA</th>
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