CYTOF CHARACTERIZATION OF THE IN VIVO LATENT HIV RESERVOIR IN BLOOD AND TISSUES

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ABSTRACT

Background. One of the major hurdles for directly phenotyping the *in vivo* latent HIV reservoir is the need to stimulate these cells *ex vivo*. As the cells are stimulated, they change expression of many cell surface markers making precise phenotyping of the latent cells difficult. We recently established and validated an approach called PP-SLIDE (Predicted Precursor as determined by SLIDE) that can effectively backtrack in time to predict the cell surface phenotype of a cell before infection (Cavrois et al 2017) using high-dimensional datasets generated by CyTOF phenotyping. This approach was implemented to chart the *in vivo* latent HIV reservoir and identify biomarkers of these cells.

Methods. The ability of PP-SLIDE to trace the phenotype of a reactivated cell to its precursor (non-stimulated) state was first confirmed in an *in vitro* model of HIV latency. Then, freshly isolated blood and lymph node cells from ART-suppressed, HIV-infected individuals from the SCOPE cohort were stimulated with PMA/ionomycin, and reactivated cells (expressing Gag) were deep-phenotyped using a 39-parameter latency-focused CyTOF panel. PP-SLIDE was used to map reactivated cells onto a T cell atlas of unstimulated cells created for each patient sample analyzed.

Results. Comparison of latent to non-latent cells revealed unique signatures associated with latency. Receptors preferentially expressed on latently-infected T cells from blood include previously described markers such as PD1, TIGIT, and OX40, as well as novel ones including homing integrin α4β1, and were predominantly amongst specific subsets of Tem. Latent cells from lymph nodes also preferentially expressed PD1, TIGIT, and OX40, and differed from latent cells in blood in that they exhibited features of resident memory cells and preferentially expressed the T follicular helper marker CXCR5 and the costimulatory molecules CD28 and ICOS. A 12-parameter sorting strategy is currently being implemented to assess to which the identified markers of latent cells enriched for intact, replication-competent HIV.

Conclusions. By simultaneously detecting the expression of 39 proteins in individual reactivated cells and mapping this information onto an atlas of unstimulated cells, we have begun to establish a high-resolution view of the types of latent cells that persist in HIV-infected individuals. Applying this method to chart the reservoir in blood and tissues of additional donors will provide a more complete picture of the nature of the persistent reservoir. Our findings thus far reveal that latent cells exhibit unique features, some of which differ between blood and tissues consistent with the notion that unique mechanisms of persistence are present in tissues.

Fig. 1. CyTOF phenotyping of immune cells

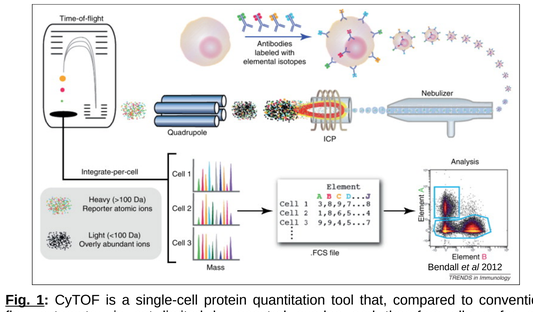


Fig. 1: CyTOF is a single-cell protein quantitation tool that, compared to conventional flow cytometry, is not limited by spectral overlap and therefore allows for more parameters to be simultaneously monitored. Cells labeled with metal-conjugated antibodies are injected into the nebulizer, become aerosolized, are reduced to single cell-containing droplets, and are directed to the ICP torch, where they are vaporized, atomized, and ionized in the plasma. The high pass optic removes the low-mass ions resulting in an ion cloud that enters the TOF mass analyzer. Ions are separated based on mass and accelerated to the detector, which measures the quantity of each isotope for each individual cell. Data can then be exported in an FCS format and analyzed.

Fig. 2. Classifying HIV-infected cells requires overcoming issue of HIV-induced remodeling

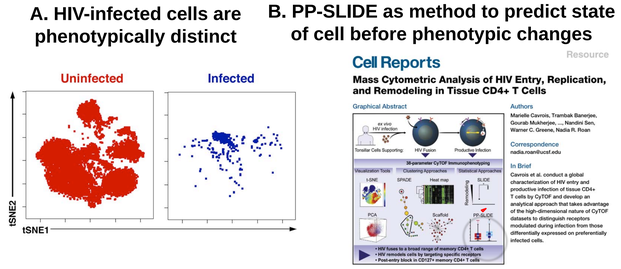


Fig. 2: (A) Uninfected and HIV-infected T cells isolated from tonsils of an uninfected donor were deep-phenotyped by CyTOF and analyzed by tSNE. The productively-infected cells (right) are largely phenotypically distinct from the uninfected T cells (left), as a result of remodeling of T cells by HIV. (B) A variety of high-dimensional analytical approaches can be used to visualize, quantitate, and account for viral-induced remodeling. One of these, PP-SLIDE, was developed as a single-cell high-dimensional analysis approach that predicts the phenotype of cells prior to cellular perturbations, in this case remodeling induced by HIV. Predictions made by PP-SLIDE were validated through cell sorting experiments. Adapted from Cavrois et al, 2017.

Fig. 3. *Ex vivo* reactivation to identify latent cells

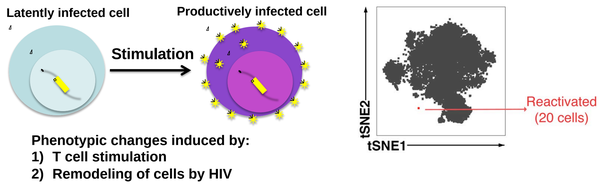


Fig. 3: (A) Direct phenotyping of the in vivo latent reservoir requires *ex vivo* stimulation in the presence of suppressive ART to reactivate the latent cells. Reactivation will induce phenotypic changes as a result of the *ex vivo* stimulation as well as remodeling of cells by HIV. (B) Visual demonstration by tSNE that reactivated cells from an HIV-infected individual are phenotypically distinct from unstimulated T cells from that individual.

Fig. 4. PP-SLIDE as a "molecular facial recognition" tool to predict the original state of changed cells

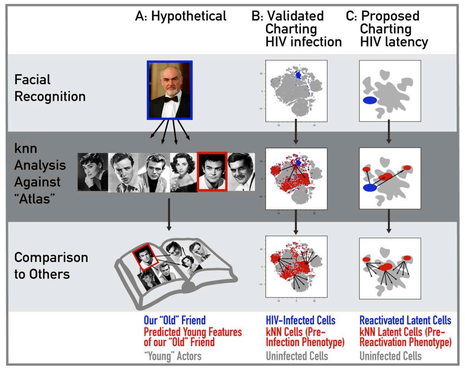


Fig. 4. PP-SLIDE, a "molecular facial recognition" tool to find k-nearest neighbors (knn), allows characterization of cells without the effects of perturbation-induced changes as a confounder. (A) In this analogy, facial recognition allows the use of a photograph of "Old" Sean Connery to identify the "Young" Sean Connery in the "Young" population, and to compare him as a young actor to hypothetical peers of his time. (B) Using a similar concept, infected cells were compared to uninfected cells without HIV-induced remodeling as a confounder. Using PP-SLIDE, the "knn cells" harboring the predicted phenotypes of cells prior to infection were identified amongst the uninfected cells (dataset from Cavrois et al, 2017). (C) To identify the original phenotypes of *in vivo* latent cells, PP-SLIDE is used to identify the knn latent cells from the non-stimulated population. These identified knn latent cells are then compared to other unstimulated cells to determine the types of cells that are preferentially latently-infected.

Fig. 5. Latency-focused CyTOF Panel

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Cell Type Identifier	Differentiation / Subset Identifier	Activation State	Homing Receptors	Others
CD19	CD45RO	CD69	CCR5	TIGIT
CD14	CD45RA	CD25	CCR6	CD30
CD3	CD62L	HLA-DR	CCR7	NFAT
CD8	CD57	CD38	CXCR4	Blimp-1
CD4	CD127	ICOS	CXCR5	Survivin
(TCRγδ)	CD27	OX40	α4β7	(CD32)
	CD103	CD28	CD49d	(CD2)
	Tbet	PD-1	(CD29)	(LAG3)
	RORγt	CTLA4	(CCR2)	(CD122)
KC57 Gag	CRTH2		(CXCR3)	(Bcl6)
Gag mix	CD95		(CCR4)	(Runx1)
bnAb mix	Foxp3			(Eomes)

Fig.5: Latency-focused CyTOF panel. Antibodies against antigens in parentheses are in alternate versions of the panel or currently under development.

Fig. 6. Proof of Concept: PP-SLIDE identifies precursors of latent JLAT clones

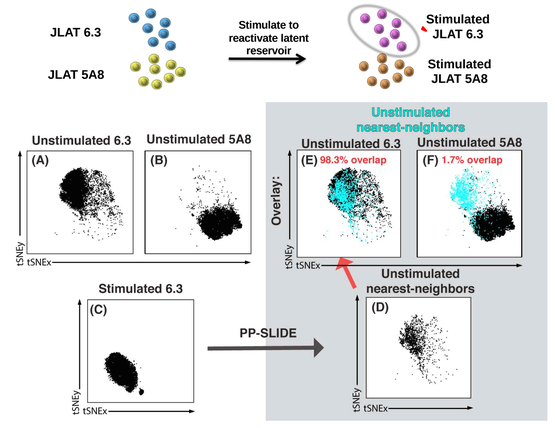


Fig. 6. Tracking activated JLAT cells to their parental clone by PP-SLIDE. *Top:* Experimental design. Unstimulated and PMA/ionomycin-stimulated JLAT clones 6.3 and 5A8 were deep-phenotyped by CyTOF, and PP-SLIDE was used to predict the precursor of stimulated 6.3. *Bottom:* t-SNE plots of unstimulated JLAT 6.3 (A), unstimulated JLAT 5A8 (B), and PMA/ionomycin-stimulated JLAT 6.3 (C). PP-SLIDE was used to identify the knn latent cell of each reactivated 6.3 cell in a merged file of unstimulated 6.3 and 5A8 cells. Note the marked overlap of the knn latent cells (D) with the unstimulated JLAT 6.3 (E, 98.3% overlap) and not JLAT 5A8 (F, 1.7% overlap), validating the ability of PP-SLIDE analysis of CyTOF-phenotyped cells to identify the pre-stimulation phenotype of reactivated latent cells.

Fig. 7. Single-cell detection of rare reactivated cells from ART-suppressed, HIV-infected individuals

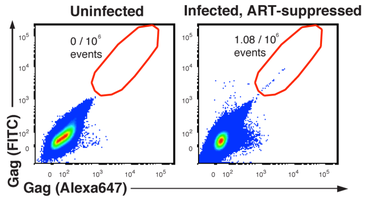


Fig. 7. Dual Gag staining to detect reare ex vivo reactivated cells from HIV-infected individuals. CD4+ T cells from an uninfected (*left*) or an HIV-infected, ART-suppressed (*right*) SCOPE individual were stimulated for 40 hours with PMA/ionomycin in the presence of suppressive ART. Cells were dually stained for Gag and analyzed by FACS. Results are gated on live CD3+CD8- T cells. Similar results were observed by CyTOF (not shown).

Fig. 8. Map of latent cells portrayed by PD-1 expression and FlowSOM-generated clusters

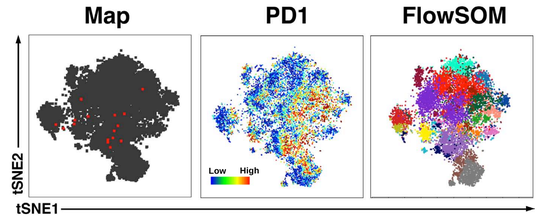


Fig. 8: Data were generated from purified memory CD4+ T cells isolated from an HIV-infected, ART-suppressed woman in SCOPE. *Left:* The knn latent cells, corresponding to the phenotypically most similar unstimulated cell to each reactivated cell, are shown in red, placed onto a tSNE map of unstimulated memory CD4+ T cells from the same sample. *Middle:* Same tSNE plot colored in heatmap fashion based on PD-1 expression. Note regions of higher PD-1 expression overlap with locations of knn latent cells. *Right:* The unstimulated memory CD4+ T cells of the "atlas" were clustered by the clustering algorithm embedded in the FlowSOM visualization program. Each color corresponds to a different cluster. Some clusters harbor latent cells while others do not.

Fig. 9. Participant-tailored sort strategy to enrich for latent cells

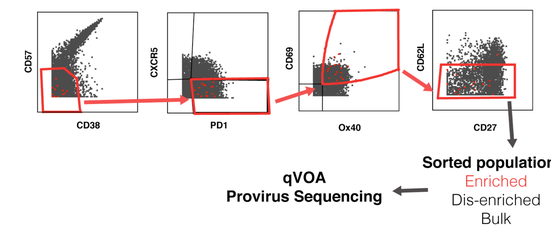


Fig. 9: Memory CD4+ T cells from blood of an HIV-infected ART-suppressed woman was subjected to analysis by CyTOF and PP-SLIDE, and markers found to be enriched and dis-enriched on the knn latent cells were used to design a sort strategy for this patient sample. Once sorted, the enriched, dis-enriched, and bulk population of cells will be subjected to qVOA and provirus sequencing (collaboration with S. Palmer, U. of Sydney) to assess the extent to which the strategy enriched for intact / replication-competent HIV.

Fig. 10. Phenotypic properties of latent cells from lymph node aspiration

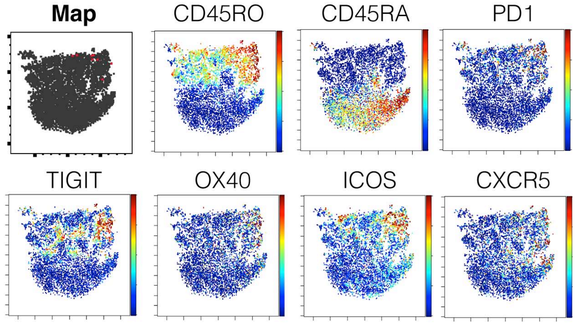


Fig. 10: Total T cells isolated from lymph nodes (via fine needle aspiration) of an HIV-infected ART-suppressed man was subjected to analysis by CyTOF and PP-SLIDE. The map in the upper left shows the knn latent cells in red superimposed onto the map of total unstimulated CD4+ T cells. The knn latent cells in this donor are present amongst memory (CD45RO+CD45RA-) cells, and tend to express PD1, TIGIT, Ox40, ICOS, and CXCR5.