AN IMMUNOCYTOCHEMISTRY METHOD TO INVESTIGATE THE TRANSLATIONALLY ACTIVE HIV RESERVOIR

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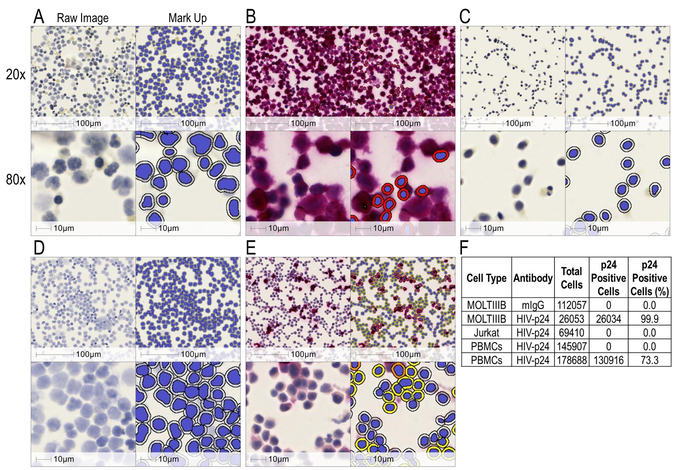
ABSTRACT

Background. Despite the success of combination antiretroviral therapies (cART) to suppress HIV replication, HIV persists in a long-lived reservoir that can give rise to viral rebound upon cART cessation. The active reservoir consists of infected cells that continue to transcribe HIV and produce viral protein even in the presence of cART. These transcriptionally and translationally competent HIV-infected cells likely contribute to chronic immune activation in people living with HIV and are implicated in rebound viremia upon cART cessation. Methodologies to quantify the active reservoir are needed.

Methods. We developed an automated immunocytochemistry (ICC) assay coupled with computational image analysis to detect and quantify intracellular Gag capsid protein (CA). For this purpose, we cytospinned fixed cells onto microscopy slides and used an automated stainer with antibodies against CA. Stained slides were digitized with a slide scanner, after which nuclear staining was used to enumerate total cells and chromogenic signal to quantify the percentage of CA-positive cells. We used qPCR, digital ELISA and flow cytometry to quantify plasma viral loads (pVL) and CA to validate ICC-CA with established assays.

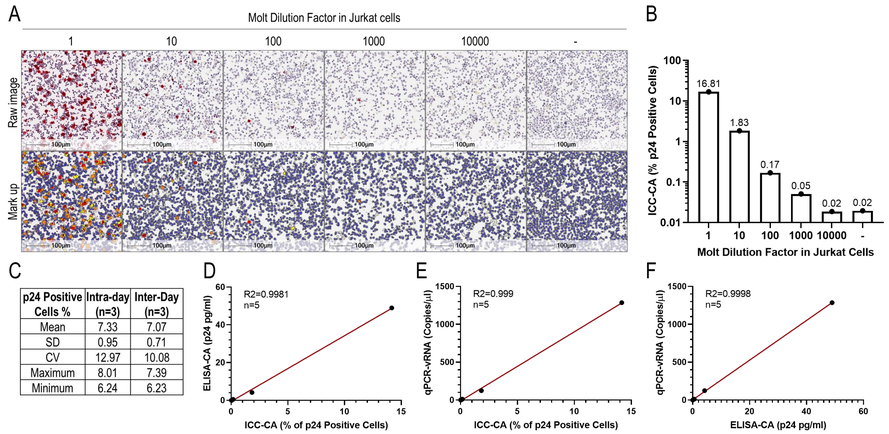
Results. We determined the sensitivity of the ICC-CA assay using the MoltIIIB cell line spiked into uninfected Jurkat cells in limiting dilutions and assayed the specificity of the staining with peripheral blood mononuclear cells (PMBCs) from HIV-seronegative donors or after in vitro infection with an HIV laboratory strain. We then evaluated the application of this assay with mouse and non-human primate animal models to detect HIV-1 p24- or SIV p27-containing cells in PBMCs, respectively. The frequency of intracellular CA positive cells from both animal models' PBMCs corresponded with the pVL and cell-associated CA. This ICC-CA method allowed us to quantify the activity of small molecule targeted activators of cell kill (TACK) in eliminating CA-expressing cells.

Conclusions. We developed and validated an ICC assay to investigate the active HIV-1 reservoir. Efforts are ongoing to multiplex markers to further characterize the active HIV-1 reservoir and provide a benchmark towards a functional HIV-1 cure.



Cell Type	Antibody	Total Cells	p24 Positive Cells	p24 Positive Cells (%)
MOLTIIIB	mIgG	112057	0	0.0
MOLTIIIB	HIV-p24	26053	26034	99.9
Jurkat	HIV-p24	69410	0	0.0
PBMCs	HIV-p24	145907	0	0.0
PBMCs	HIV-p24	178688	130916	73.3

Figure 1. Detection of HIV-p24 (CA) in human chronically infected cell lines and in vitro infected primary cells by immunocytochemistry, ICC-CA. A) MoltIIIB cells stained with anti-mIgG or **B)** with anti-p24 antibodies. **C)** Jurkat cells stained with anti-p24 antibodies. **D)** Uninfected and **E)** in vitro infected human PBMCs stained with anti-p24 antibodies. **F)** Proportion of HIV-p24 positive cells detected by ICC-CA. Representative raw images and relative computational mark up are shown. Top and bottom panels represent 20x and 80x magnification, respectively.



p24 Positive Cells %	Intra-day (n=3)	Inter-Day (n=3)
Mean	7.33	7.07
SD	0.95	0.71
CV	12.97	10.08
Maximum	8.01	7.39
Minimum	6.24	6.23

Figure 2. Evaluation of ICC-CA sensitivity and correlation to standard viral assays. A) MoltIIIB spiked in Jurkat cells in serial dilutions and **B)** relative frequency of HIV-p24 positively stained cells. **C)** Coefficient variation, CV (%) for MoltIIIB spiked in Jurkat cells at 7%: intra- and inter-day CV. Correlation of ICC-CA with **D)** ELISA-based detection of cell-associated p24 and **E)** with qPCR-based quantification of cell-associated HIV-RNA. **F)** Correlation of ELISA-CA and qPCR-HIV-RNA detection. Representative raw images and relative computational mark up are shown.

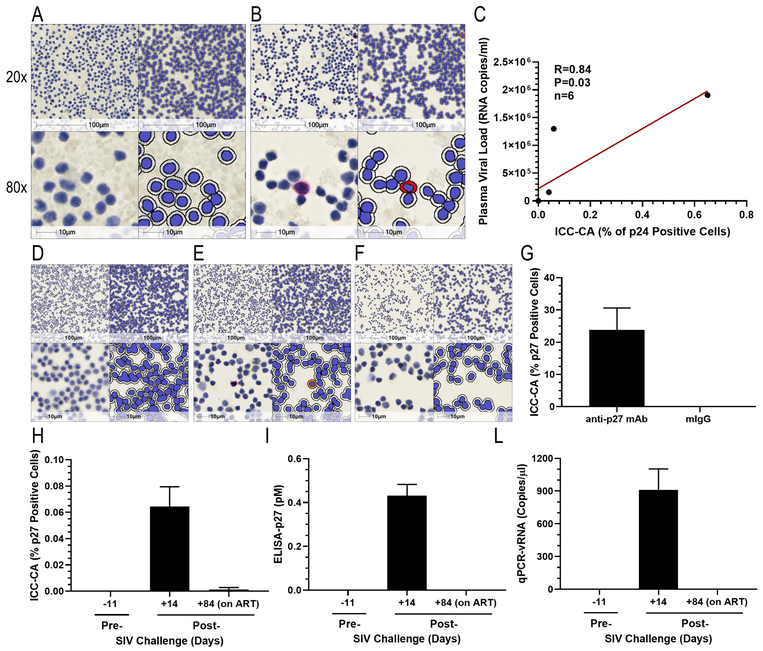


Figure 3. Application of ICC-CA to PBMCs derived from mouse and non-human primate models. ICC-CA mediated detection of HIV-p24 in retro-orbital bleeds of **A)** uninfected, **B)** infected mice and **C)** relative correlation with plasma viral loads. SIV-p27 detection in Rhesus macaques' PBCMs obtained **D)** 11 days before, **E)** 14 days and **F)** 84 days post challenge (ART initiated at day 56). **G)** Frequency of SIV-p27 by ICC-CA in SIV in vitro infected Rhesus macaque PBMCs. Quantification of **H)** cell-associated SIV-p27 by ICC-CA, **I)** ELISA and **L)** cell-associated SIV-RNA by qPCR (analyses conducted on two animals). Representative raw images and relative computational mark up are shown. Top and bottom panels represent 20x and 80x magnification, respectively.

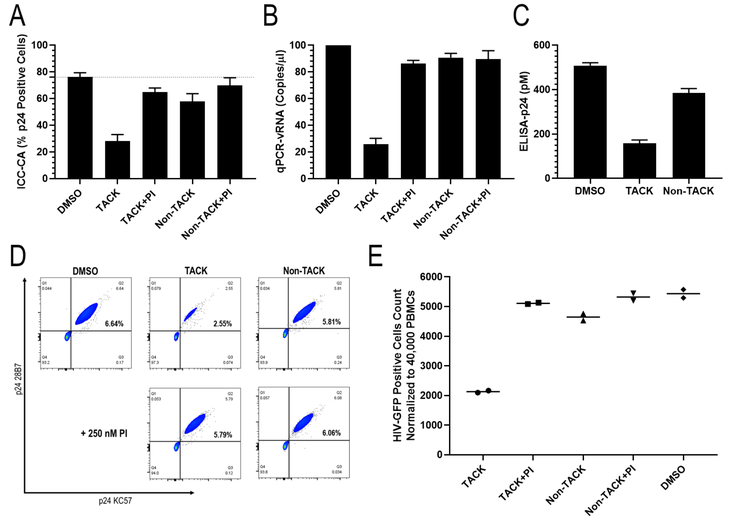


Figure 4. Application of ICC-CA to quantify the activity of small molecule targeted activator of cell kill, TACK. PBMCs infected with a replication incompetent HIV-GFP reporter virus and treated with antivirals were used to quantify **A)** the frequency of HIV-p24 positive cells by ICC-CA, **B)** cell-associated viral RNA by qPCR, **C)** cell-associated p24 by ELISA, **D)** cell-associated p24 by flow cytometry and **E)** cell-associated GFP by microplate reader. Non-TACK, TACK inactive NNRTI; PI, HIV protease inhibitor.

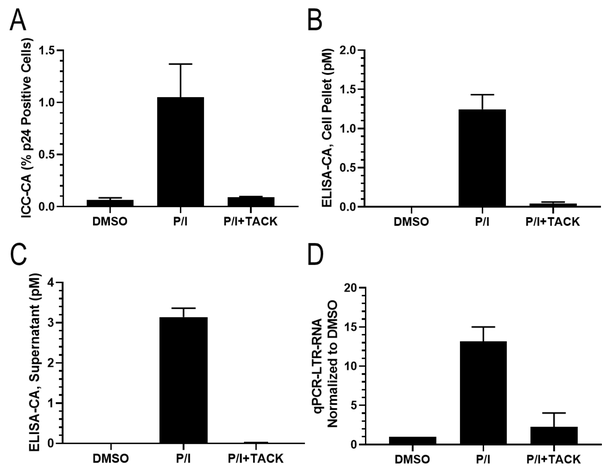


Figure 5. Application of ICC-CA to quantify TACK activity in PBMCs from HIV seropositive donors on ART. Quantification of HIV-p24 positive cells by **A)** ICC-CA, **B)** ELISA (cell pellet), **C)** ELISA (culture supernatant) and **D)** cell-associated HIV-RNA by qPCR. P/I, PMA/Ionomycin.

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