HIV latency is reversed by ACSS2-driven histone crotonylation

Guochun Jiang[1], Don Nguyen[1], Nancie M. Archin[2], Steven A Yukl[2], Gema Méndez-Lagares[1], Yuyang Tang[1], Maher Elsheikh[1], George R. Thompson III[1], Dennis J. Hartigan-O'Connor[1], David M. Margolis[2], Joseph K. Wong[3], and Satya Dandekar[1,*]

[1] Department of Medical Microbiology and Immunology, University of California, Davis, CA 95616, USA.

[2] Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, 27599 USA

[3] Department of Medicine, University of California, San Francisco, and San Francisco VAMC, San Francisco, 94121 USA

[*]Corresponding Author: Satya Dandekar, email: sdandekar@ucdavis.edu

Department of Medical Microbiology and Immunology

UC DAVIS, Davis CA 95616

Running title: Histone crotonylation regulates HIV latency

Materials and Methods

Western Blot Analysis One million cells were treated with LRAs for 4-18 hours. Whole cell lysates were prepared using RIPA buffer. Total histone proteins were prepared using EpiQuik Total Histone Extraction Kit (Epigentek, Farmingdale NY). Following antibodies were bought from Cell Signaling (Boston, MA) for Western blot to detect protein expression under different drug conditions: ACSS2 (or AceCS1, cat number 3658), Cyclin T1 (cat number 8744), CDK9 (cat number 2316), p-CDK9 (cat number 2549), NF-κB (cat number 8242), GAPDH (cat number 2118) and total H3 (cat number 9717S). Antibodies for H3K4Cro (cat number PTM-527, PTM Biolabs, Chicago, IL), H3K18Cro (cat number ab195475, Abcam, Cambridge, United Kingdom), H3K27Me3 (cat number, Cell Signaling, Boston, MA), H3K4Ac (cat number 7627, Cell Signaling, Boston, MA) and H3K18Ac (cat number 9675, Cell Signaling, Boston, MA) were used to measure histone modifications.

Chromatin Immunoprecipitation (ChIP)

J-Lat A1 cells (2.5 to 5 million) were utilized in ChIP assays with antibodies as above, such as anti-H3K18Ac, anti-H3K4Cr, anti-H3K27Me3, anti-H3K4Ac, anti-SIRT3 (cat number 2627S, Cell Signaling, Boston, MA), and anti-SIRT1 (cat number 9475, Cell Signaling, Boston, MA) antibodies. ChIP assays were performed using SimpleChIP Kit from Cell Signaling as per the manufacturer's recommendations. Samples were cross-linked using formaldehyde and 150-1000 bp fragments were made using Micrococcal Nuclease digestion and 7 cycles of 30 sec on/30 sec off sonication. Antibodies were used to probe respective proteins. Purification of DNA was performed using the SimpleChIP Kit Protocol. Purified ChIP DNA was used in SYBR Green qPCR using ChIP primers specific for HIV Long Terminus Repeat (LTR).

Real-Time PCR Analysis

HIV gene expression was measured using real-time PCR in J-Lat A1 cells treated with LRAs. Total nucleic acid was extracted using RNeasy Kit, genomic DNA digested using DNase I and total RNA reverse transcribed using Superscript III to synthesize cDNA.

HIV gene expression was determined by quantitative real-time PCR (ABI ViiA 7 detector) using the following primer/probe set: for J-Lat A1 cells, primer 1: 5'-GGAGCGACCATCTTCTTCA-3', primer 2: 5'-AGGGTGTCGCCCTCGAA-3', probe 5'-FAM CTACAAGACCC GCGCCGAGGTG TAMRA-3'.

Sample processing and metabolic analysis. Lumen contents were collected from intestinal loops of control (n=9 from 3 SIV negative animals) and 10 week SIV-infected(n=9 from 3 SIV positive animals) rhesus macaques (**Supplementary Table 2**). The frozen lumen contents were sent to Metabolon, Inc. in Durham NC and stored at -80 ^{o}C until sample processing for metabolic analysis. Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis with Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS).

Quantitative analysis of synergy of latency reversing agent combinations We applied the Bliss independence model as implemented by Laird et al and Jiang et al to test for synergy when NaCro treatment was combined with PEP005(*3, 4*). For Na-Cro (drug x) and PEP005 (drug y) we used the equation $fa_{xyP}=fa_x+fa_y-(fa_x)(fa_y)$, where fa_{xyP} represents the predicted fraction affected by the combination of drug x and drug y given the observed effects of drug x (fa_x) and drug y (fa_y) used individually and fa_{xyO}= the observed effect when x and y were tested together. Calculation of fa_x for patient CD4$^+$ T cells followed the approach of Laird et al for intracellular HIV RNA : fa_x = *(HIV*

RNA copies with drug x – background copies with control)/ (HIV RNA copies with PMA –background copies with control). In cases where one or more experimental drug conditions resulted in RNA expression exceeding the PMA condition, we imputed the highest HIV RNA value in that experiment to represent the denominator for calculation of fa_x. With this model , $\Delta\ fa_{xy} = fa_{xyO}$ (the observed fraction affected by the drug combination) - fa_{xyP} (the predicted fraction affected by the drug combination) provides an indication of synergy ($\Delta\ fa_{xy} > 0$) , additive effect (Bliss independence) ($\Delta\ fa_{xy} = 0$) or antagonism ($\Delta\ fa_{xy} < 0$). Statistical significance was determined using a one tailed ratio paired t-test comparing fa_{xyO} and fa_{xyP} for using GraphPad Prism 6.

Figure Legends

Supplementary Figure 1. Na-Cro reactivated latent HIV in HIV latent model of U1 cells.

(**A**, **B**) Na-Cro addition to U1 promonocytic cells reactivated HIV expression in a dose dependent manner as measured by RT-qPCR (**A**) (n=5) or by p24 ELISA assays (**B**) (n=3). **, p<0.01; ***, p<0.001 compared with control treatment. The data were analyzed with One-way ANOVA.

Supplementary Figure 2. Na-Cro synergistically reactivates latent HIV in J-Lat A1 cells with other LRAs. (A) Na-Cro synergistically reactivated the latent HIV in combination with PEP005 (n=3), Bryostatin-1 (n=4), or SAHA (n=3) in J-Lat A1 cells. Bliss independence was analyzed from at least three independent experiments to determine the synergy among combined compound treatment using the data in Figure 3A after normalized with PMA treatment. **(B)** Pre-treatment with Na-Cro promotes SAHA reactivation of latent HIV in U1 cells. The latent U1 cells were pre-treated with 30 mM Na-Cro followed by 500 nM SAHA or pre-treated with 500 nM SAHA followed by 30 mM Na-Cro. The cells were collected for RNA extraction and RT-qPCR analysis of HIV transcription with real-time PCR (n=3). ***, p<0.001 compared with SAHA treatment. The data were analyzed with One-way ANOVA.

Supplementary Figure 3. Evaluation of immune activation in the T cells during ACSS2 induction via treatment of Na-Cro. PBMCs isolated from healthy donors (n=5) were treated with 100ng PMA plus 2μg/ml Ionomycin or 40 mM Na-Cro for 24 or 72 hours. Then, the cells were collected and stained for anti-CD3, anti-CD4, anti-CD8, anti-CD69, anti-HLA-DR and anti-PD-1 antibodies. And the percentage of total T cells, $CD4^+$ T cells, or $CD8^+$ T cells was determined by flow cytometry. **, $p<0.01$; ***, $p<0.001$; ***, $p<0.001$ compared with control treatment. The data were collected from PBMCs of 5 healthy donors and analyzed with One-way ANOVA.

Supplementary Figure 4. Reactivation of latent HIV by Na-Cro alone or in combination with PEP005. Primary $CD4^+$ T cells isolated from HIV+ patients (n=6) under ART were treated with 100 ng/ml PMA plus 2 μM ionomycin, 12 nM PEP005, 40 mM Na-Cro, or 12 nM PEP005 combined with 40 mM Na-Cro for 6 hours. Viral transcripts from total RNA was analyzed by RT-ddPCR with primers targeting initiation (**A**), elongation (**B**), or full transcription (**C**) of the HIV genome.

Supplementary Figure 5. Na-Cro synergistically reactivates the latent HIV with PEP005 in primary $CD4^+$ T cells isolated from patients under suppressive ART. Observed Fa_{xy} or predicted Fa_{xy} was determined by Bliss independence analysis from patient samples as described in the Supplementary Material and Methods (n=6).

Supplementary Figure 6. Na-Cro promotes vorinostat (SAHA) reactivation of latent HIV in primary $CD4^+$ T cells isolated from patients under suppressive ART. **(A)** Na-Cro enhanced vorinostat (SAHA) reactivation of latent HIV. Primary $CD4^+$ T cells were isolated from frozen PBMCs of HIV+ patients (n=6) under ART. Then, 0.5-1 million $CD4^+$ T cells were treated with 100 ng/ml PMA plus 2 μM ionomycin, 500 nM vorinostat (SAHA), 40 mM Na-Cro, or 500 nM vorinostat combined with 40 mM Na-Cro for 6 hours. The cells were collected for total RNA purification. As Na-Cro was more effective on the initiation of HIV transcription after short time treatment, viral transcripts from total RNA was analyzed by RT-ddPCR with primers targeting initiation of the HIV genome.

(B) No synergy was observed between Na-Cro and SAHA in reactivation of latent HIV in patient samples. Bliss independence was similarly analyzed as in Figure 4 to determine the synergy from patient samples (n=6).

References:

1. M. D. George, E. Reay, S. Sankaran, S. Dandekar, Early antiretroviral therapy for simian immunodeficiency virus infection leads to mucosal CD4+ T-cell restoration and enhanced gene expression regulating mucosal repair and regeneration. *Journal of virology* **79**, 2709-2719 (2005).
2. G. Jiang, C. Santos Rocha, L. A. Hirao, E. A. Mendes, Y. Tang, G. R. Thompson, 3rd, J. K. Wong, S. Dandekar, HIV Exploits Antiviral Host Innate GCN2-ATF4 Signaling for Establishing Viral Replication Early in Infection. *mBio* **8**, (2017).
3. G. M. Laird, C. K. Bullen, D. I. Rosenbloom, A. R. Martin, A. L. Hill, C. M. Durand, J. D. Siliciano, R. F. Siliciano, Ex vivo analysis identifies effective HIV-1 latency-reversing drug combinations. *The Journal of clinical investigation* **125**, 1901-1912 (2015).
4. G. Jiang, E. A. Mendes, P. Kaiser, D. P. Wong, Y. Tang, I. Cai, A. Fenton, G. P. Melcher, J. E. Hildreth, G. R. Thompson, J. K. Wong, S. Dandekar, Synergistic Reactivation of Latent HIV Expression by Ingenol-3-Angelate, PEP005, Targeted NF-kB Signaling in Combination with JQ1 Induced p-TEFb Activation. *PLoS pathogens* **11**, e1005066 (2015).

Suppl. Table 1. Characteristics of HIV-1-infected participants in this study

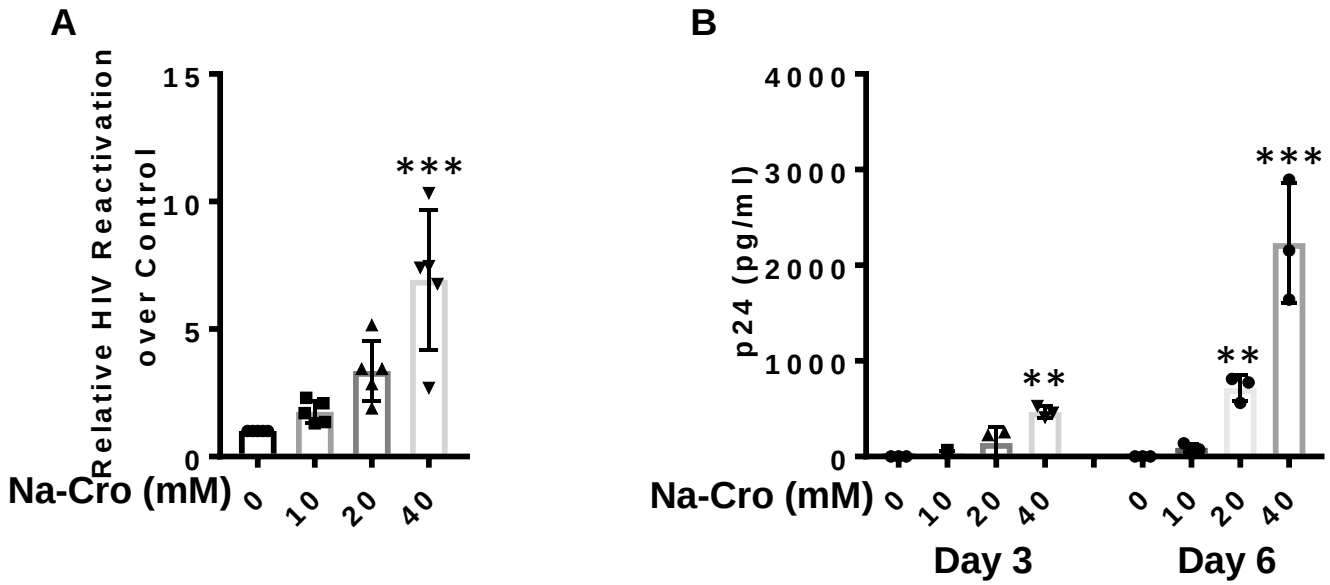
Pt ID	Age	Sex	VL	CD4 Count	ART	Years under ART
1	62	M	<20	788	Epzicom and Neviripine	>10
2	59	M	<20	682	Atripa	>10
3	58	M	<20	502	Triumeq	>17
4	59	M	<20	885	Triumeq	> 8
5	46	F	0	465	Stribild	>12
6	54	M	<20	267	Truvada and Dolutegravir	>15
7	51	M	0	749	Stribild	>14
8	22	M	0	942	Stribild	>3
9	57	M	<20	744	Atripla	>14
10	31	M	<20	995	Tivicay and Odefsey	>2
11	32	M	<20	402	Triumeq	>3
12	60	M	<20	742	Triumeq	>19
UNC-1	32	M	<40	666	Odefsey	>3
UNC-2	59	M	<20	733	Atazanavir, Ritonavir, FTC, Tenofovir	>6
UNC-3	28	M	<40	701	Darunavir, Ritonavir, Truvada	>5
UNC-4	45	F	<20	1050	Atripla	>6
UNC-5	25	M	<40	535	Triumeq	>1

Suppl. Table 2. Animals for metabolic analysis

Animal ID	SIV	Wks post infection	Viral load/ml
39114	Negative	0	NA
37467	Negative	0	NA
39343	Negative	0	NA
39296	Positive	10	79738
36701	Positive	10	49036
39238	Positive	10	242970

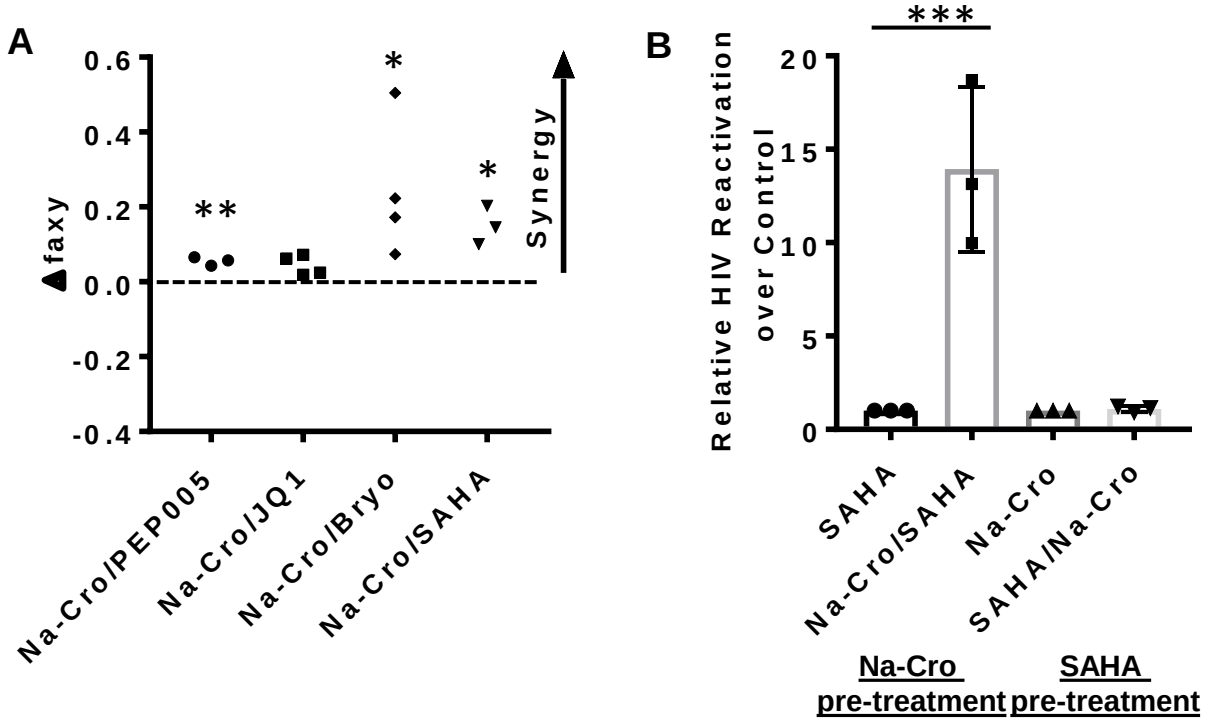
Suppl. Figure 1.

Jiang et al.,

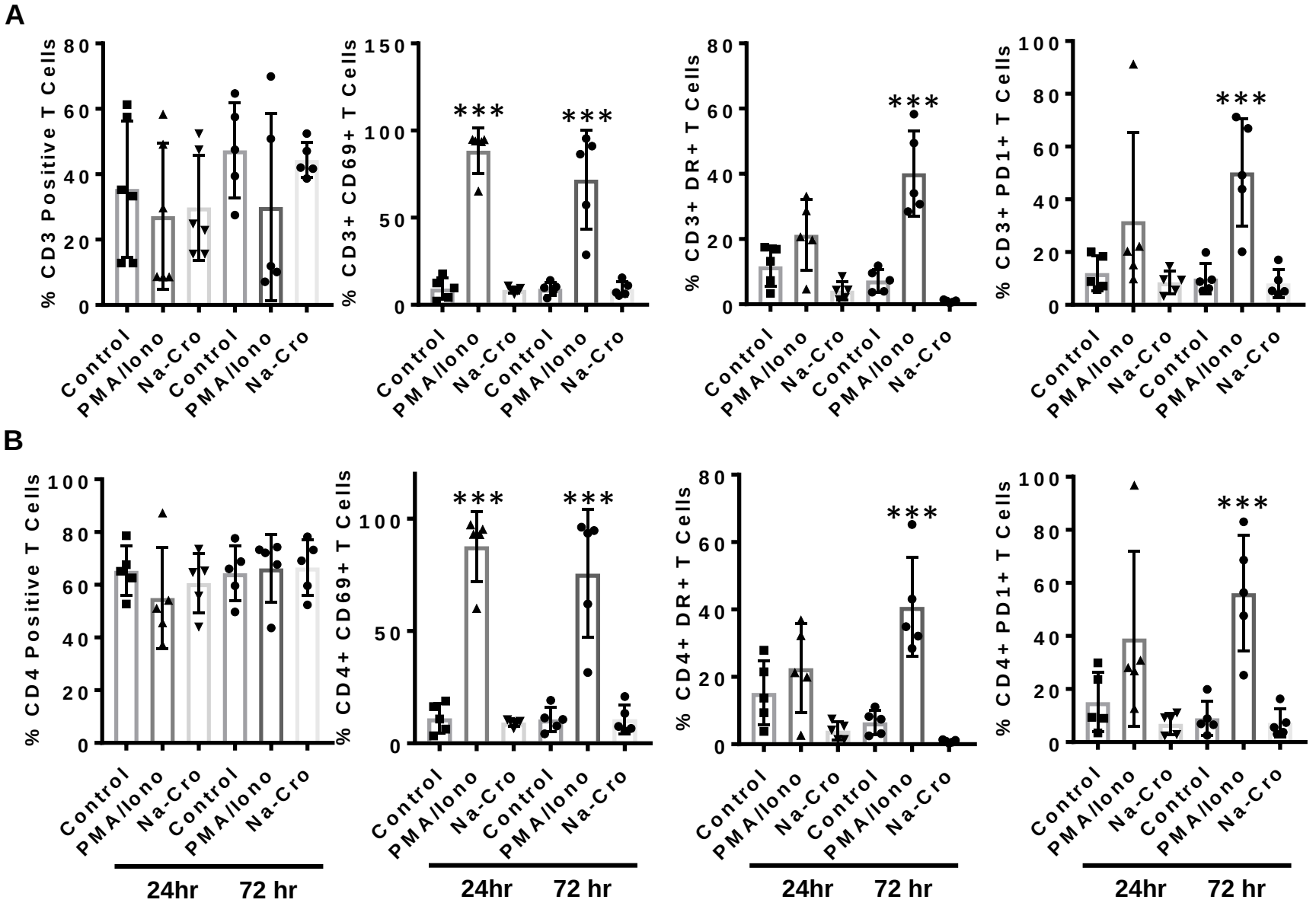


Suppl. Figure 2.

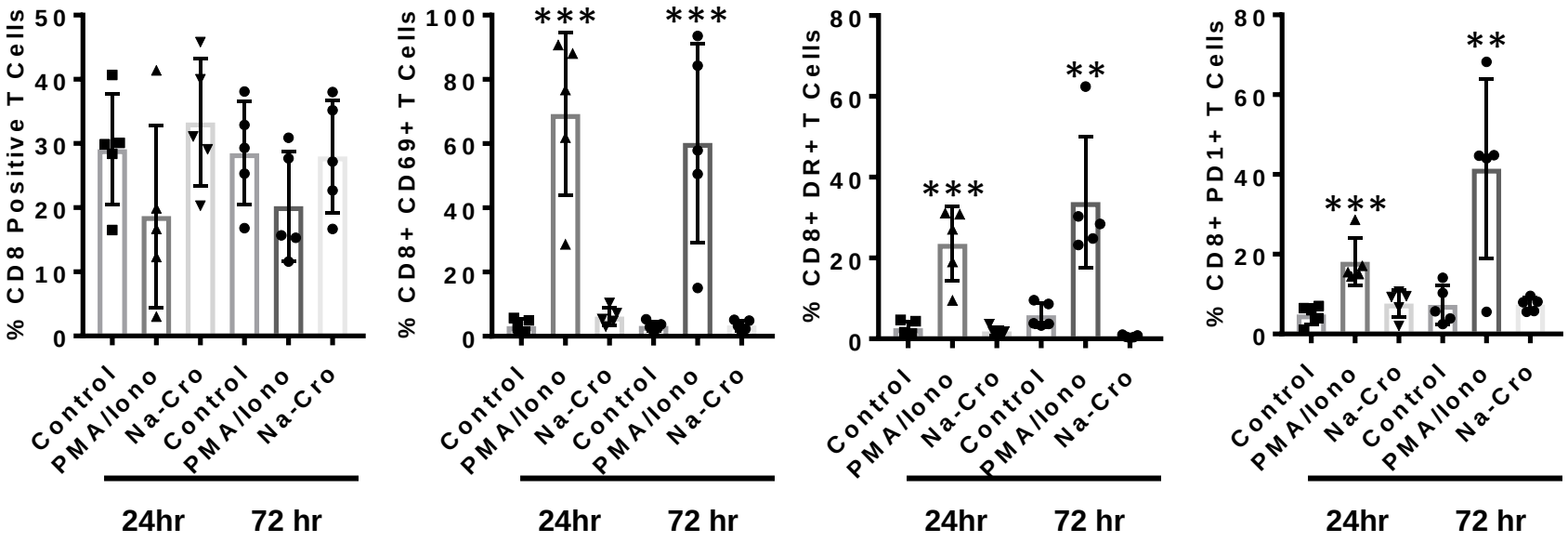
Jiang et al.,



Suppl. Figure 3. **Jiang et al.,**

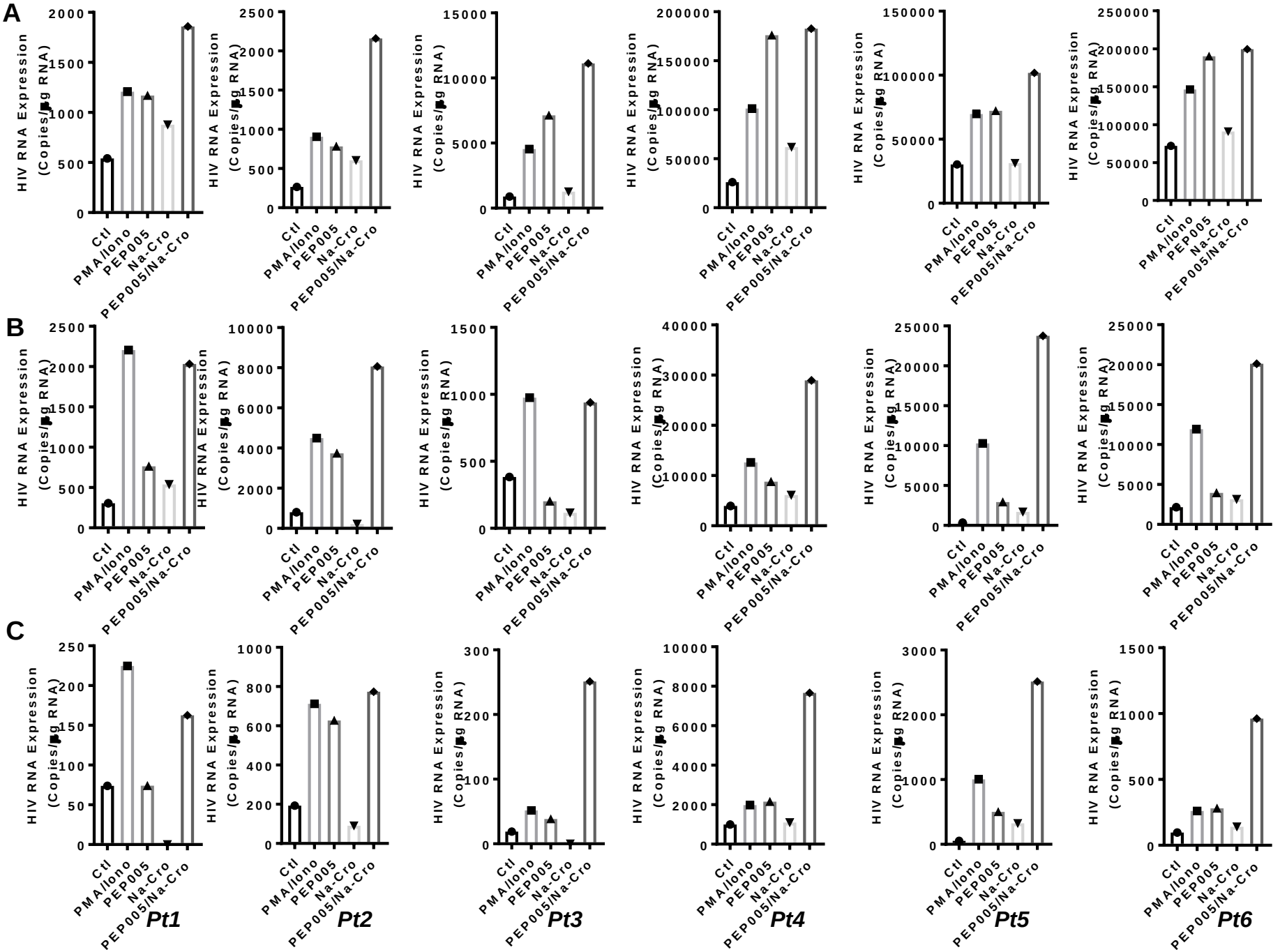


C


% CD8 Positive T Cells
50
40
30
20
10
0
% CD8+ CD69+ T Cells
100
80
60
40
20
0
***
***
% CD8+ DR+ T Cells
80
60
40
20
0
***
**
% CD8+ PD1+ T Cells
80
60
40
20
0
***
**
Control
PMA/Iono
Na-Cro
Control
PMA/Iono
Na-Cro
24hr
72 hr

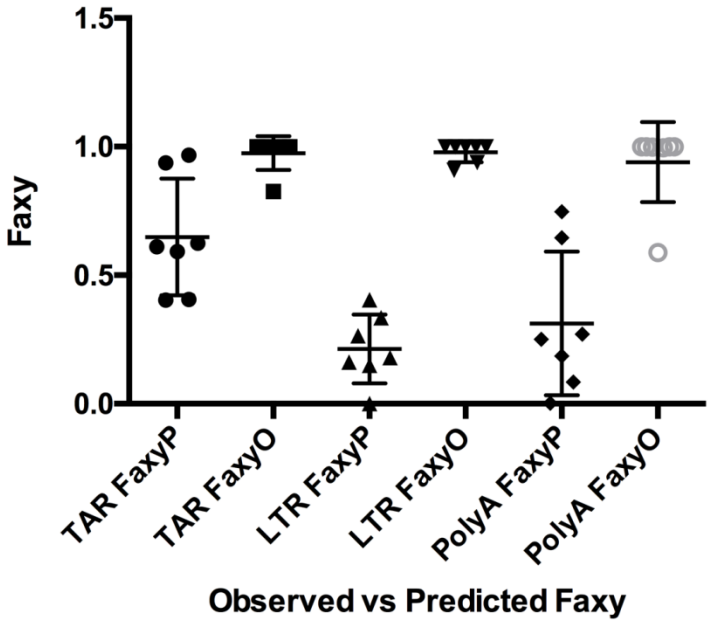
Suppl. Figure 4.

Jiang et al.,



Suppl. Figure 5.

Jiang et al.,



Suppl. Figure 6.

Jiang et al.,