INCREASED BIRC3 TRANSCRIPTION IS ASSOCIATED WITH INCREASED HIV PRODUCTION

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•**Background-**ACH-2 cells are A3.01 cells containing a single proviral HIV genome. Unstimulated ACH-2 cells produce low levels of HIV. Prior to stimulation, surface staining of HIV Env identifies two ACH-2 populations, one that stains for Env and p24 and one that does not. With TNF stimulation >90% of virus production is from the Env^{brt} population while an Env^{dim} population arises from the Env^{-} population. We compared the cellular transcriptome of Env^{brt} to Env^{dim} and A3.01 cells before and after stimulation with TNF.

•**Methods-**ACH-2 cells were dual stained with PG9 and VRC07 to identify the envelope bright, dim and null populations and bulk sorted at 0, 3, 6, 9 and 24h post-stimulation. A3.01 cells were sorted similarly. Cells were lysed and then frozen. Total RNA was extracted, poly-adenylated RNA purified, fragmented and then reversed transcribed using random hexamers. Illumina ready libraries were generated and sequenced by paired-end HiSeq 4000 2x75 reads. Changes that were 2-fold different with a P<0.005 were defined as significant. Individual protein levels were determined using chemiluminescent antibody arrays.

•**Results-**ACH-2 and A3.01 transcriptomes were similar post-stimulation in many ways. In both ACH-2 and A3.01 cells RELA and NFKB1 message were elevated prior to and did not change with TNF stimulation. Increased NFKB2 and NFKBIA in both A3.01 and ACH-2 cells 3h after stimulation and increases in RELB and CD82 suggested rapid activation and transport of NFκB to the nucleus and early NFκB driven phosphorylation and transport of AP-1 to the nucleus. There were also differences. HIV RNA read frequency was significantly higher in ACH-2 bright than in ACH-2 dim cells and absent in A3.01 cells at all times. These changes were associated with differences in NFκB associated gene transcription. Most strikingly BIRC3 transcripts, which plays an important role in NFκB activation, were significantly increased in Env^{brt} compared to A3.01 cells post-stimulation. This change was not observed in the Env^{dim} population. Increases in BIRC-3 protein at 1 hour and phosphorylation of AP-1 at 30m were confirmed by protein array.

•**Conclusions-**These data indicate low level transcription of HIV RNA prior to stimulation in Env^{brt} cells are associated with higher level BIRC3 after stimulation. Increased transcription of BIRC3 may contribute to increased HIV production in these cells.

Introduction: Identification of HIV-infected CD4 T cells and characterization of the HIV infection on CD4 T cells has been a major research goal for >20 years. The advent of transcriptomics makes it possible to assess the effect of infection on CD4 mRNA levels both *in vitro* and *in vivo*. The average lifespan of an HIV-infected CD4 T cells *in vivo* is estimated to be 2.2 day; interpretation of both *in vitro* and *in vivo* transcriptomic data is made difficult by the inability to identify what stage of infection individual cells are. ACH-2 cells are A3.01 cells containing LAV proviral DNA. Unstimulated, these cells produce low levels of HIV (Fig, 1) and a fraction of these cells express intra-cellular p24 and surface expression of envelope prior to stimulation (Fig. 2). Addition of TNFα results in dramatically increased levels of HIV production after a 6 hour incubation period. Analysis of these cells allows longitudinal characterization of the transcriptome of HIV infected cells.

Methods: ACH-2 and A3.01 cells were obtained from the AIDS reagent program. VRC07 G54W was conjugated using a mini-biotin XX protein labeling kit; PG9 was directly label with and alexa fluor 690 labeling kit, both from Invitrogen. Strepavidin AF487 was used to label the biotin conjugate. Intracellular p24 staining was done using KC57 FITC obtained from Beckman Coulter. HIV production was followed using Perkin-Elmer p24 ELISA kit. Cellular BIRC3 and phospho- c-Jun levels were determined using R&D human phopho-kinase and human NFκB array kits as described by the manufacturers protocol.
Cells were incubated in R-10 and stimulated with 100U of TNF/ml. At appropriate time points cells were centrifuged at 400xg at 4C, washed, stained on ice for surface expression of envelope, and sorted, based on envelope staining, into ice cold fetal calf serum (FCS) using a BD FACS Diva. 20,000 cells were collected for each population at each time point. Sorted cells were immediately spun down, lysed with RNAzol and then frozen. Total RNA was extracted, poly-adenylated RNA purified, fragmented and then reversed transcribed using random hexamers. Illumina ready libraries were generated and sequenced by paired-end HiSeq 4000 2x75 reads.
Differential gene analysis was done using DESeq2. The significance threshold was set at a Padj of ≤0.005 and a >2x increases in reads. For HIV read-through analysis paired end reads were aligned to the human genome (HG38) and a reference HIV sequence with BLAST+. Reads that aligned to both the human genome and 5' and 3' HIV LTRs in a non-overlapping manner were identified as "chimeric" reads. To identify the HIV insertion site, the alignment coordinates from the alignment to HG38 were compared to a reference of gene and exon locations.
Genes associated with specific transcription factors were identified using the TRRUSTv2 database (Han, H et al. Nucleic Acids Res. (2018) 46(D1, D380-D386),

Results:

Productively-infected ACH-2 are defined by surface expression of envelope

Fig. 1

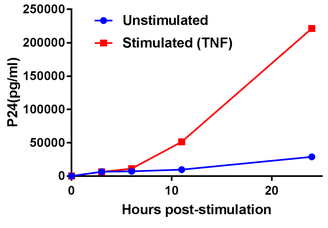

Fig. 1 100U/ml of TNFα were added to ACH-2 at 0 time and aliquots of media were taken at 3, 6, 11 and 24 hours after addition of TNF. Cells were centrifuged at 400xg and supernatant frozen until assayed for P24 by ELISA.

Fig. 2

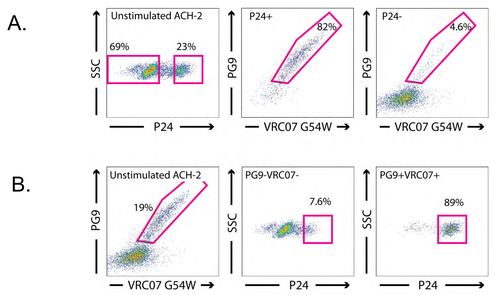

Fig. 2 Unstimulated cells, surface stained for envelope with PG9 and VRC07 G54W and then permeabilized and stained for intracellular P24. A.) Staining showing the proportion of P24+ and P24- cells stained with PG9 and VRC07. B.) Staining showing the proportion of cells surface stained envelope+ and envelope- which stained for P24.

Fig. 3

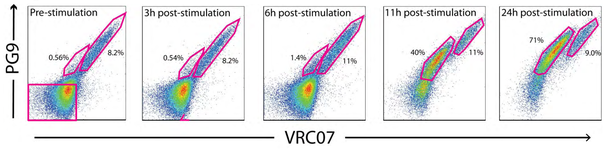

Fig. 3. 100U/ml of TNFα were added to ACH-2 cells at time 0 and staining of cells were followed longitudinal. Staining of cells with pre-titered amounts of PG9 and VRC07 G54W showed envelope staining of 3 different population with i) an increase in staining intensity of the envelope bright population and ii) cells which did not stain (null staining) and iii) cells which stained dimly for envelope to an intermediate staining population with TNFα stimulation.

Fig. 4

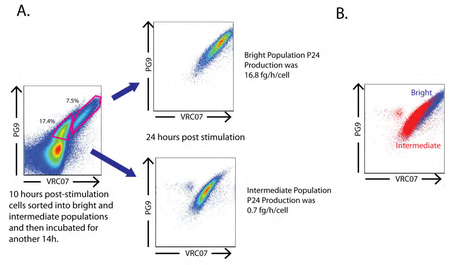

Fig. 4 ACH-2 cells were stimulated with 100U/ml TNFα and incubated for 10h. A) Cells were then surface stained with PG9 and VRC07 G54W, these cells were then sorted into envelope intermediate and bright populations and incubated for an additional 14 hours in the presence of 100U/ml TNFα. Media was then taken, cells were spun down, media P24 content determined by ELISA and the rate of production of virus/h/cell determined. Virus production by envelope bright cells was 20 fold greater than by envelope dim cells. B) To assure that cells maintained the same phenotype during this experiment cells from the bright and intermediate population were stained at the end of the 14h incubation. Overlays of the staining pattern of the 2 cell types are shown.

TNFα stimulation results in a rapid increase in proviral DNA transcription which precedes increased envelope expression.

Fig. 5 A. B.

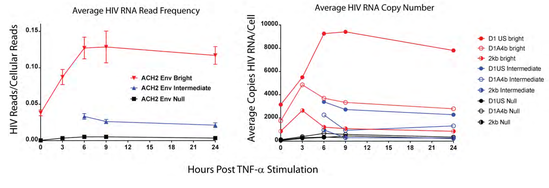

Fig. 5 Quantitation of intracellular viral copy number using next generation sequencing and RT PCR. For each method, approximately 20,000 cells were collected for each cell phenotype and time point. A) Cell were stained and sorted into FCS and immediately frozen. Upon thawing total cellular RNA was extracted with RNAzol, poly A purified, fragmented and then amplified using random hexamers. Illumina libraries were generated and sequenced by paired-end HiSeq 4000 2x75 reads. HIV reads were quantitated as a fraction of total cellular HIV RNA and plotted longitudinally. B) Cells were stained and sorted into cold R-2, lysed and RCAS IS added to each sample prior to cell lysis and RNA purification.. HIV RNA copy numbers were determined by RT PCR using primer and probe sets specific for unspliced HIV RNA, D1A4b HIV RNA and D4A7 (2kb) RNA.

TNF-α stimulation results in a rapid increase in NFκB1 controlled genes in both A3.01 and ACH-2 cells.

Fig 6

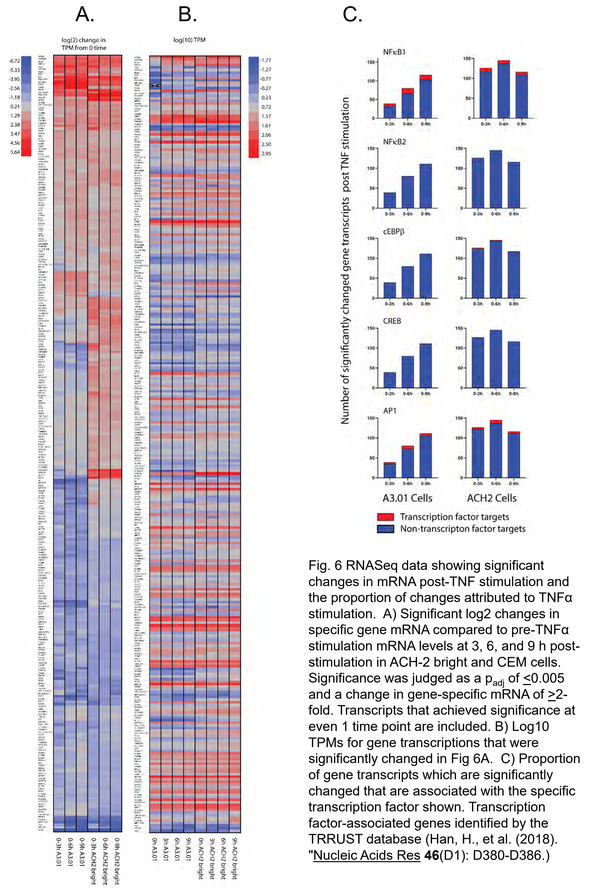

Fig. 6 RNASeq data showing significant changes in mRNA post-TNF stimulation and the proportion of changes attributed to TNFα stimulation. A) Significant log2 changes in specific gene mRNA compared to pre-TNFα stimulation mRNA levels at 3, 6, and 9 h post-stimulation in ACH-2 bright and CEM cells. Significance was judged as a p_{adj} of ≤0.005 and a change in gene-specific mRNA of ≥2-fold. Transcripts that achieved significance at even 1 time point are included. B) Log10 TPMs for gene transcriptions that were significantly changed in Fig 6A. C) Proportion of gene transcripts which are significantly changed that are associated with the specific transcription factor shown. Transcription factor-associated genes identified by the TRRUST database (Han, H., et al. (2018). "Nucleic Acids Res **46**(D1): D380-D386.)

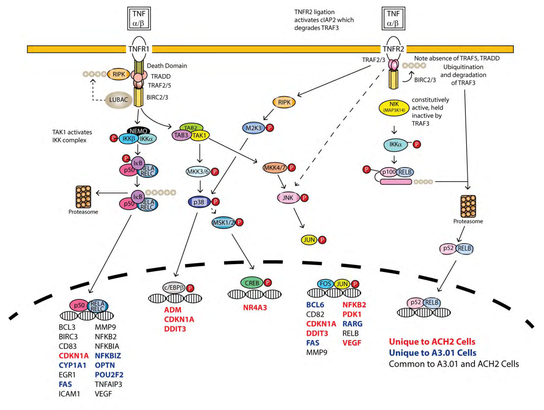

Fig. 7. TNFR1 and TNFR2 pathways with associated transcription factors shown. mRNA transcripts which significantly changed with TNF stimulation are shown. Transcripts which are common to both A3.01 and ACH-2 are shown in black. RNA species which were only significantly changed in A3.01 are shown in blue. RNA species which only changed in ACH-2 bright cells are shown in red.

TNF Stimulation of ACH-2 cells increase c-Jun Phosphorylation and BIRC3 synthesis

Fig. 8

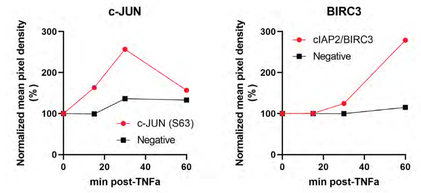

Fig. 8 Longitudinal production of BIRC3 and c-Jun Phosphorylation by bulk ACH-2 cells after stimulation with TNFα.

TNFAIP3, BIRC-2 and BIRC-3 transcript frequency suggest envelope expressing ACH-2 cells are at a higher level of activation pre-stimulation than A3.01 cells.

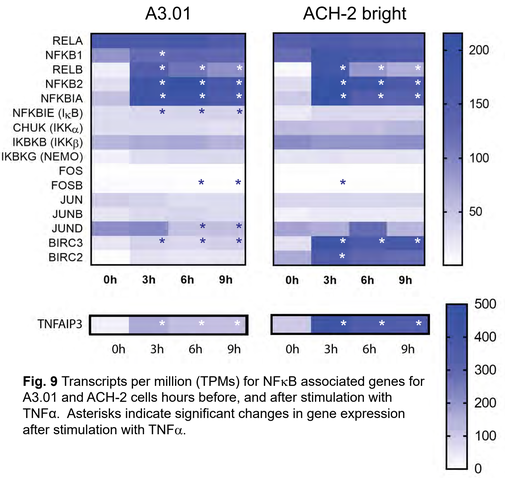

Fig. 9 Transcripts per million (TPMs) for NFκB associated genes for A3.01 and ACH-2 cells hours before, and after stimulation with TNFα. Asterisks indicate significant changes in gene expression after stimulation with TNFα.

HIV read through data suggest that the majority of ACH-2 cells that produce virus have more than one HIV integration site.

Fig. 10

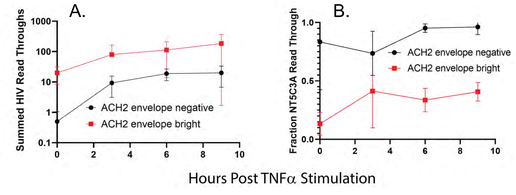

Fig. 10 Average HIV-read throughs from ACH-2 bright and ACH-2 null cells A) Total HIV read throughs for 20,000 ACH-2 bright cells and null cells. Error bars represent SD. B) The fraction of total HIV read throughs associated with the NT5C3A intron, the primary insertion point for proviral HIV DNA in ACH2 envelope negative and positive cells. N=6 for all data. Error bars represent SD.

Conclusions

1. Even though increases in measurable HIV production by ACH-2 cells do not occur in the first 6 h after TNF stimulation, increases in intracellular HIV RNA occur within the first 3h.
2. A fraction of ACH-2 express envelope on their cell surface before TNF stimulation. These cells continue to express envelope at a high level after TNF stimulation and are responsible for the vast majority of HIV production by ACH-2 cells.
3. TNFα driven NFκB stimulation of A3.01 and ACH-2 cells occurs primarily through the canonical pathway.
4. Transcriptome patterns suggest that ACH-2 envelope bright cells express higher levels of intracellular NFκB prior to stimulation than A3.01 cells. These differences are maintained after TNFα stimulation and may explain increased HIV production by these cells.
5. HIV read-through analysis suggest that envelope bright ACH-2 cells contain more than 1 HIV insertion site. This super-infection of AHC2 cells may be responsible for the increased production of HIV by envelope-bright ACH-2 cells.