Figures and figure supplements

Diverse fates of uracilated HIV-1 DNA during infection of myeloid lineage cells

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Figure 1. Ex-ddPCR and Ex-Seq determine the uracil content of the HIV DNA copies. (A) After infection of immune cells with the single-round VSV-G pseudo-typed virus HIV\(^{NL4.3(VSVG)}\), total DNA is isolated and either digested by UNG to degrade uracil containing DNA or mock digested to provide a measure of total amplifiable DNA in the sample. The output (Frac U\(_{\text{DNA}}\)) represents the fraction of amplified DNA copies containing at least one uracil on each DNA strand. Signal is normalized to a genomic reference copy standard (RPP30) that does not contain uracil. (B) Activated, resting CD4\(^+\) T cells and MDMs were infected in vitro with HIV\(^{NL4.3(VSVG)}\) virus and the uracil content was measured 3 days post-infection (dpi) using primers that targeted the gag region. The data (± UNG digestion) are shown as scatter plots and histograms. (C) Normalized coverage of the HIV\(^{NL4.3(VSVG)}\) genome-positive strand in Excision-Seq (Ex-Seq) libraries prepared from total cellular DNA at 7 days post-HIV infection. (D) Fraction of the reads in panel C that contained uracil (Frac U). (E) Discordant read pairs between HIV and human DNA present in Ex-Seq libraries prepared from total cellular DNA at 7 days post-infection with HIV\(^{NL4.3(VSVG)}\) virus. The number of discordant reads obtained by Ex-Seq in the absence and presence of UNG digestion are shown as white and black bars.

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Figure 1—figure supplement 1. Profiling enzyme activities and dNTP pool levels in immune target cells of HIV. Extracts from each indicated cell type were obtained as described in Methods. (A) Deoxyuridinetriphosphate hydrolase (dUTPase) activity was measured by monitoring the hydrolysis of dUTP to dUMP via PEI-cellulose TLC. Specificity was determined using a potent dUTPase inhibitor [compound 26 in Priet et al. (2005)]. (B) SAMHD1 triphosphohydrolase activity was determined by C18 RP-TLC-based assay using 8-3H-labeled dGTP as the substrate. Specificity for SAMHD1 was determined using the inhibitor pppCH2dU. The mobilities of the substrate (dGTP) and product nucleoside (dG) are marked. (C) Endogenous uracil DNA glycosylase (hUNG) activity (combined hUNG1 and hUNG2) was determined using a fluorescein-labeled DNA substrate that shows an increase in fluorescence upon uracil excision. Specificity for hUNG activity was determined by addition of the uracil DNA glycosylase inhibitor protein (UGI). (D) Apyrimidinic endonuclease (APE1 or 2) activity was measured using a fluorescein-labeled duplex DNA containing a single abasic site that increases in fluorescence upon endonuclease cleavage. (E) dUTPase activity. (F) SAMHD1 activity. (G) hUNG activity. (H) APE activity. (I) Measurement of deoxyribonucleotide (dNTP) pool levels were determined by an LC-MS method. (J) MDMs contain a high ratio of dUTP/TTP ratio compared to resting and activated CD4+ T cells. Abbreviations: rCD4+, resting CD4+ T cell; aCD4+, PHA activated CD4+ T cell; MDM, monocyte-derived macrophage. See Supplemental Methods for further details and references describing these assays. Number of experimental replicates (n = 3) and errors are reported as mean ± SD.

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Figure 1—figure supplement 2. In vitro generated calibration curves for evaluating uracil content in DNA amplicons and single-round HIV infections of cultured MDMs. (A) ddPCR primer and probe locations relative HIV genome (HIVNL4.3(VSVG)). (B) Generation of uracil-containing duplex DNA of increasing length and ratio of dUTP/TTP was achieved by in vitro DNA polymerization using Taq polymerase in the presence of various ratios of dUTP/TTP. These DNA standards (79 to 471 bp) were analyzed by the Ex-ddPCR method as indicated. (C) The fraction of the DNA amplicons that contained detectable uracil (Frac U) increased with DNA amplicon length as well as the dUTP/TTP ratio used in the initial DNA synthesis. (D) In single-round infections of a mixed population of MDMs with HIVNL4.3(VSVG), the copy number of early, middle and late viral cDNAs were measured over a 30-day culture period using primers specific to different regions of the viral genome (see above). The copy number is normalized to one million MDM target cells. (E) Uracil content was measured in each HIV DNA population using Ex-ALU-gag nested qPCR. Number of experimental replicates (n = 1–3) and errors are reported as mean ± SD.
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Figure 2. Uracils arise from high dUTP/TTP not APOBEC-catalyzed cytosine deamination. (A) Culturing a mixed GFP+/GFP– MDM cell population with media supplemented with 5 mM thymidine resulted in a 14-fold decrease in the fraction of proviral copies that contained uracil. Uracil content was measured using Ex-ALU-gag nested qPCR with their statistical significance level (*p<0.05). (B) Mixed MDM cultures supplemented with 5 mM thymidine showed a five-fold increase in provirus copy number as measured using ALU-gag nested qPCR. Number of experimental replicates (n = 4–5) and errors are reported as mean ± SD.

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The following source data is available for figure 2:

Source data 1. Ex-ALU-gag qPCR for provirus and uracil detection in MDMs cultured with standard media or media supplemented with 5 mM Thymidine (Figure 2A).

DOI: 10.7554/eLife.18447.007
A3G

HIV

SF162(CCR5)

- + + + +

dpi 1 7 14 30

GAPDH

A3A

PBMC

MDM

Figure 2—figure supplement 1. APOBEC3G (A3G) and APOBEC3A (A3A) are poorly expressed in a mixed population of MDMs. Western blots established that the expression levels of APOBEC3A (A3A) and A3G in a mixed population of MDMs over a 30-day culture period after infection with HIV$^\text{SF162(CCR5)}$ were consistently low or undetectable. The detection of A3G and A3A in PBMCs and their absence in HEK 293T (293) cells indicates the antibody is specific for each protein.

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Figure 3. MDMs consist of two distinct cell populations with respect to viral infection. (A) Flow cytometry analysis of MDMs infected with replication-deficient HIV^{NL4.3(VSVG)} and replication competent HIV^{SF162(CCR5)} viruses. Expression of virally encoded GFP was only observed in 12–15% cells over a 30-day time-period. (B) After cell sorting according to GFP fluorescence, ddPCR was used to measure the copy number of HIV^{NL4.3} reverse transcription intermediates (RTIs) in GFP− (white bars) and GFP+ (green bars) MDMs. Level of statistical significance (**p<0.05). (C) ALU-gag nested qPCR measurement of the HIV^{NL4.3(VSVG)} provirus copy number in sorted GFP− and GFP+ MDMs. (D) Measurement of HIV^{SF162(CCR5)} provirus copy number and uracil content over the course of a 30-day multi-round infection. (E) ELISA measurements of viral p24 protein levels in GFP− and GFP+ MDMs over the course of a 30-day multi-round infection. Number of experimental replicates (n = 3–5) and errors are reported as mean ± SD.
DOI: 10.7554/eLife.18447.009
The following source data is available for figure 3:
Source data 1. Ex-ALU-gag qPCR for measurement of reverse transcription intermediates (RTIs) and provirus content in GFP sorted MDMs (Figure 3B,C). DOI: 10.7554/eLife.18447.010
Figure 3—figure supplement 1. Uracilation is independent of multiplicity of infection (MOI). MDMs were infected with HIVNL4.3(VSVG) using MOIs of 0.1, 1 and 10. (A) The viral gag copy number increases with MOI. (B) The MOI does not significantly affect the fraction of viral gag copies that contained uracil. Number of experimental repetitions (n = 3) and errors are reported as mean ± SD.
DOI: 10.7554/eLife.18447.011
The sorted populations of in vitro infected GFP− and GFP+ MDMs are highly pure. MDMs were infected with HIV

\( ^{NL4.3} \)

by spinoculation and then sorted using GFP fluorescence after 7 days. The purity of each sorted population (GFP− and GFP+) was assessed by flow cytometry. Each population was greater than 99% pure. (A) Flow cytometry analysis of purified GFP− population. (B) Flow cytometry analysis of purified GFP+ population.

DOI: 10.7554/eLife.18447.012
Figure 3—figure supplement 3. Three different viral strains show a similar uracilation profile with in vitro infected MDMs independent of the differentiation regimen. (A) The kinetics and absolute levels of virus output as measured by p24 ELISA is indistinguishable for MDMs infected with...
HIV$^{SF162/(CCR5)}$ and HIV$^{BAL/(CCR5)}$. (B) Provirus copy number and uracil content at dpi = 3 are very similar in MDMs infected with HIV$^{SF162/(CCR5)}$, HIV$^{NL4.3/(VSVG)}$ or HIV$^{BAL/(CCR5)}$ (MOI = 0.1). Proviral copy numbers were measured using ALU-gag nested qPCR and the uracil content by Ex ALU-gag nested qPCR. (C) Infection of MDMs obtained from peripheral blood monocytes of three donors produces equivalent levels of uracilated HIV$^{NL4.3/(VSVG)}$ DNA as judged by ddPCR analysis of the GFP copies. (D) The fraction of viral GFP copies that contain uracil is similar for all three donors as determined using Ex-ddPCR (ns, not significant). (E) Purified monocytes isolated from bulk PBMCs were differentiated to MDMs in vitro using the adherence method, or by addition of M-CSF or GM-CSF. The differentiation regimen did not significantly affect viral GFP expression in MDMs infected with HIV$^{NL4.3/(VSVG)}$, or (F) proviral DNA copy number as determined by ALU-gag nested qPCR, or (G) the fraction of viral DNA copies that contained uracil as determined by Ex ALU-gag nested qPCR. Number of experimental replicates (n = 2–3) and errors are reported as mean ± SD.

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GFP expression from HIV\textsuperscript{NL4.3} infected MDM populations does not strongly correlate with CD14 and CD16 expression. MDMs (dpi = 3) were initially distinguished by their characteristic FSC and SSC and then assessed for their expression levels of CD14 and CD16 markers. (A) MDMs were sorted according to GFP expression using FACS and then double stained for CD14 and CD16 expression before analysis by flow cytometry. (B) The relative CD14 and CD16 marker expression levels in mixed and GFP-sorted MDM populations were quantified using flow cytometry.

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Figure 4. hUNG2 uracil excision activity are required for efficient pre-integration restriction. Effect of transient overexpression of nuclear hUNG2 and Ugi on copies of viral early reverse transcription intermediates (RTIs) and proviruses. (A) Experimental scheme for hUNG2 and Ugi overexpression in MDMs. (B) Copies were determined by qPCR using specific primers for early RTIs and provirus. (C) Fraction of viral RTIs and provirus that contained detectable uracil under conditions of hUNG2 depletion (Ugi) and overexpression (hUNG2). ND; Not detectable. (D) Western blot analysis of the vpr deletion virus HIV\textsuperscript{NL4.3(Dvpr)} revealed no detectable Vpr. Western blot analysis to detect nuclear (hUNG2) and mitochondrial (hUNG1) hUNG isoforms detected oscillation in the hUNG2 levels over the course of a 7-day multi-round infection. At day 7, the HIV\textsuperscript{NL4.3(Dvpr)} shows abundant hUNG levels while hUNG2 is depleted in the wild-type infection. (E) Measurements of provirus copy number in a mixed population of GFP\textsuperscript{−} and GFP\textsuperscript{+} MDMs infected with HIV\textsuperscript{NL4.3(VSVG)} (wt) and HIV\textsuperscript{NL4.3(Dvpr)}. (F) Ex-ALU-gag nested qPCR measurements of the fraction of uracilated proviral copies in a mixed population of GFP\textsuperscript{−} and GFP\textsuperscript{+} MDMs infected with HIV\textsuperscript{NL4.3(VSVG)} (wt) and HIV\textsuperscript{NL4.3(Dvpr)} at 3 days post-infection. Level of statistical significance (*p<0.05). Number of experimental replicates (n = 4–5) and errors are reported as mean ± SD.

Figure 4 continued on next page
Figure 4 continued

DOI: 10.7554/eLife.18447.015

The following source data is available for figure 4:

Source data 1. Ex-ALU-gag qPCR for measurement of reverse transcription intermediates (RTIs), provirus and uracil content in MDMs transfected with plasmids encoding hUNG2, Ugi or empty control (Figure 4B,C).

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Figure 5. Impact of uracilation and cytokines on viral DNA and RNA sequences. (A) Experimental protocol. (B) Quantitative reverse transcriptase-PCR (qRT-PCR) was used to determine the copy numbers of extracellular viral RNA in cell supernatants from sorted GFP+ (green bars) and GFP− (white bars) MDMs with and without cytokine stimulation. Number of experimental replicates (n = 4) and errors are reported as mean ± SD. (C) Summary of mutations from limiting-dilution clonal sequencing of extracellular viral RNAs obtained from infected GFP− MDMs under different growth conditions. The mutation frequencies (point mutations per total nucleotides sequenced) were obtained from a ~500 bp amplicon of the env gene. ND; not detected. (D) Representative env sequences of extracellular viral RNAs produced by GFP− sorted MDMs. The top sequence is for the HIVSF162(CCR5) strain used to infect the MDMs (HIVSF162_env_ref). Boxed regions at the protein and nucleotide sequence level show the mutation spectrum within the CD4-associated and co-receptor binding sites. (E) Breakdown of point mutations for viral RNA produced from infected MDMs and proviral DNA from infected T cells (see text).

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The following source data is available for figure 5:

Source data 1. qPCR measurement of gag copies (Figure 6B), provirus copies (Figure 6C) and uracil content (Figure 6D) in GFP sorted and cytokine-treated MDMs.

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Figure 6. Effects of cytokine stimulation on viral transmission in GFP sorted MDM populations. (A) Experimental approach. (B) MDMs infected with HIVSF162(CCR5) were sorted into GFP+ (green bars) and GFP− (white bars) populations and analyzed with respect to gag copy number (dps; days post-stimulation). (C) Provirus copy numbers for each MDM population were measured using ALU-gag nested qPCR at 3 dps. (D) The fraction of proviral DNA copies containing uracil was measured using Ex-ALU-gag nested qPCR. ND; Not Detected. (E) Viral growth kinetics of sorted MDM populations. (F) Levels of virus in culture supernatants of GFP+ and GFP− MDM producer cells were measured using p24 ELISA. (G) Viral supernatants from each MDM treatment were normalized to p24 levels and used to infect naïve CEMx174 target cells. Provirus copies were measured using ALU-gag qPCR. The results are normalized to one million target cells. In all cases, the number of experimental replicates (n = 4–5) and errors are reported as mean ± SD.

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The following source data is available for figure 6:

Source data 1. qPCR measurement of gag copies (Figure 6B), provirus copies (Figure 6C) and uracil content (Figure 6D) in GFP sorted and cytokine treated MDMs.

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Figure 7. Peripheral blood monocytes and alveolar macrophages contain high levels of uracil in HIV DNA. (A) Resting CD4+ (rCD4+) T cells and monocytes were purified by negative bead selection from bulk PBMCs obtained from six ART-suppressed individuals. Total HIV pol DNA was quantified using Ex-ddPCR. Copy numbers were normalized to the human RPP30 gene to give an estimate of HIV copies/10^6 cells. Limit of detection (LOD) = 5 copies/10^6 cells. (B) The uracilated fraction of HIV pol DNA copies derived from monocytes and rCD4+ T cells was measured using Ex-ddPCR. Total genomic DNA was also isolated from matched, cryopreserved PBMCs and bronchial alveolar macrophages (AM) obtained from a single donor both pre-ART (green circle) and post-ART (yellow circle), (**p<0.001). Donor 4 had no detectable uracil (pol) and was excluded from this plot. (C) Infection of MDMs by HIV-1 and possible fates of uracilated viral DNA products (see text). Level of statistical significance (**p<0.001). Each experimental replicate is shown. Number of experimental replicates (n = 1–3) and errors are reported as mean ± SD.

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Figure 7—figure supplement 1. Purity and detection of HIV DNA in isolated cell populations from ART suppressed individuals. Resting CD4+ (rCD4+) T cells and monocytes were isolated from six ART suppressed individuals by negative selection. The results for donor 1 are shown in the figure panels. (A) Monocytes were stained for the specific monocyte markers CD14 and CD16. (B) The purity of the monocyte preparations was determined by negative staining for the T cell marker CD3. (C) To determine if the purified resting CD4+ T cells contained contaminating activated CD4+ T cells, we stained for CD4 and the activation markers CD25/CD69/HLA-DR. (D) The purity of the purified monocyte and rCD4 cells from each patient is indicated. The HIV pol copies were determined by ddPCR and are expressed as an average and standard deviation (SD). When possible, two or three independent ddPCR measurements were taken. (E) NL4.3 plasmid was serially diluted over 100,000-fold to evaluate the detection limit of the ddPCR assay for HIV pol. (F) Dilution series of human genomic DNA with measurement of the RPP30 copy number by ddPCR. This series was used to determine the load limit for total DNA that can be accurately measured in a ddPCR reaction. (G) Measurement by ddPCR of HIV pol copy number in a background of genomic DNA obtained from 250,000 uninfected PBMCs. (H) Uracils are not detected in genomic DNA (RPP30 gene) of uninfected monocytes or T cells. Using RPP30 primers, Ex-ddPCR was performed on genomic DNA isolated from monocytes of healthy donors (open circles) or ART patients (gray circle) as well as rCD4+ T cells from ART patients. (I) Ex-ddPCR was used to determine that uracil is only found in HIV DNA isolated from monocytes and not rCD4+ T cells of HIV-infected patients. The shown data are for donor ID 2 (Supplementary file 4). For visualization, the histograms for the RPP30 Ex-ddPCR data have been down scaled by a factor of ~10^4 to facilitate comparison with the low abundance pol positive droplets. The insets show the raw scatter plots of the pol positive droplets that were used to generate corresponding histograms for each isolated cell population. The pol-positive droplets for the ddPCR and Ex-ddPCR experiments are shown in red and blue, respectively. The histograms for the pol-positive droplets follow the same color key. Number of experimental repetitions (n = 2–9) and errors are reported as mean ± SD.

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