**SUPPORTING DIGITAL CONTENT for**

**Lack of viral control and development of combination antiretroviral therapy escape mutations in macaques after bone marrow transplantation**

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**Supplementary Methods and Materials**

**Administration of cART, and collection of peripheral blood and tissue samples**

Combination antiretroviral therapy was administered to animals once or twice daily, as described previously [1] and in Figure S1. Peripheral blood was collected by venipuncture up to twice a week as previously described [2]. Gastrointestinal (GI) biopsy surgeries were performed prior to SHIV challenge, and 2–4 weeks, 9–11 weeks, 23–29 weeks, 43–45 weeks, and 62–68 weeks post-SHIV challenge, where applicable. GI biopsy samples were processed into single cell suspensions as previously described [1, 2].

**Plasma viral load and ELISA measurements from blood**

Viral RNA was isolated from plasma from peripheral blood collected in EDTA, reverse-transcribed, and unspliced SHIV RNA was measured by Taqman as previously described [3]. SIVmac239 whole virus and HIV-1 SF162 gp120-specific antibody titers were measured by ELISA and analyzed as previously described [4]. Briefly, endpoint titers were determined as the reciprocal of the highest serum dilution (1:100, 1:200, etc.) that resulted in an optical density reading greater than the average values obtained with SHIV-negative macaque sera plus three standard deviations.

**Flow cytometry**

Total CD4+ and CD8+ T-cell, and CD20+ B-cell counts in peripheral blood were measured by flow cytometry using the following fluorophore-antibody conjugates: FITC-CD2 Clone S5.2, PerCP-Cy5.5-CD20 Clone L27, FITC-CD3 Clone SP34-2, PerCP-Cy5.5-CD4 Clone L200, and APC-CD8 Clone SK1. For T-cell subset analyses from peripheral blood and GI biopsy-derived single-cell suspensions, the following clones were used: CD3 Ax700 Clone SP34-2, CD4 PerCp-Cy5.5 Clone L200, CD8 APC-Cy7 Clone SK1, CD28 PE-Cy5, Clone CD28.2, CD45RA FITC or V450 Clone 5H9, CD95 APC Clone DX2, CCR5 PE Clone 3A9, and CCR7 PE-Cy7 Clone 3D12. Complete blood cell (CBC) counts were used to calculate absolute cell counts for peripheral blood subsets measured by flow cytometry. CBC’s were also used to collect monocyte counts.

**Plasma viral RNA sequencing**

*Nucleic acid isolation:* Plasma was isolated from whole blood in EDTA. RNA from plasma was isolated with Qiagen’s QIAamp Viral RNA Mini kit (Cat No. 52904) and eluted into a 40 μl final volume of buffer AVE. Single stranded cDNA was prepared with Clontech Labs’ Takara Blueprint 1st strand cDNA Synthesis kit (Cat. No. 6115H) using the 3’ –most reverse primer for the targeted region. A list of the primers used is shown in Supplementary Table 2.

*Nested-PCR:* cDNA from viral RNA was then subjected to nested-PCR in an end point dilution series [5]. Triplicate dilutions of 1:1, 1:5, 1:25 and 1:125 were created for each sample. A 25 μl first round PCR was performed using the outer primers and the Kapa Biosystems’ KAPA HiFidelity Hotstart kit (Cat. No. KK2502) with the following thermocycler conditions: 95°C for 2 minutes, 35X (98°C 20 seconds, 62°C 30 seconds, 72°C 90 seconds), 72°C 5 minutes, 4°C hold. A 1 μl aliquot from the first round PCR was then subjected to a second round of PCR using the corresponding inner primer set and the same thermocycler conditions. The PCR products from all dilutions were then run on an agarose gel to identify positive reactions. If an endpoint was achieved (with some reaction being negative) the number of amplifiable templates in the starting material was then estimated using the Quality [5] web tool (<http://indra.mullins.microbiol.washington.edu/quality/>). If no endpoint was achieved the experiment was repeated using further dilutions of cDNA.

*Library production for sequencing*: Positive PCRs with ≥ 50 templates were selected for sequencing and cleaned with Zymo Research’s DNA Clean and Concentrator -5 (Cat. No. D4004). Random fragments ready for sequencing were produced for each amplicon by tagmentation using the Nextera DNA Sample Preparation kit (Cat. No. PE-945-2001) and standard protocols therein. Each library was verified on the Agilent 2100 Bioanalyzer with an Agilent 7500 High Sensitivity DNA Assay kit to confirm that the DNA fragments were in the range of 200–1000 bp with an average of about 500 bp. As part of the Nextera protocol, libraries for each sample and each amplicon were tagged with a unique sequence barcode (“MID” in Illumina’s notation). The amount of dsDNA in each library was quantified with Life Technologies’ Quant-iT Picogreen dsDNA Assay kit using the manufacturer’s standard protocol and a Turner Biosystems’ TBS-380 spectrophotometer. Libraries were then mixed in equimolar amounts for sequencing in batch.

*Sequencing and data processing*: Sequencing was performed on an Illumina miSeq using the 2×300bp paired end sequencing methodology and standard protocols. The sequences for each sample were then processed within the commercial software package Geneious (Biomatters Ltd.). Reads were first trimmed to remove all portions of all sequences in which a Q-value was <30 and then filtered to remove reads ≤ 199bp after trimming. The trimmed and filtered reads for each sample were then mapped to the SHIV-1157ipd3N4 inoculum sequence. Variant analysis was performed to identify any variants that occurred in two or more time points of the same sample at a frequency of greater than or equal to 2%. Variants within the expected regions of drug resistance were compared to known resistance mutations and only those shown in Figure 2 were identified.

**Measurement of plasma cART concentrations by mass spectrometry**

TFV, FTC, and RAL were extracted from monkey plasma using protein precipitation followed by LC-MS/MS analysis. Calibration standards and quality control samples were prepared in monkey plasma (EDTA) on the day of analysis. Fifty microliters of each plasma sample was mixed with 50 µL of a 50:50 methanol:water ratio containing isotopically-labeled internal standards (13C5-TFV,13C15N2-FTC,and d3-RAL). Then, 250 µl of methanol was added to each sample. Following vortex and centrifugation steps, 50 µL of the resulting supernatant was mixed with 450 µL of water prior to LC-MS/MS analysis. Two separate LC-MS/MS analyses were used for the analysis of all three analytes. TFV and FTC were eluted from a Waters Atlantis T3 (100 × 2.1mm, 3 µm particle size) analytical column, while RAL was eluted from a Phenomenex Synergi Polar-RP (4.6×50 mm, 4 µm particle size) analytical column. An API-5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) was used to detect all analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 5–10,000 ng/mL for each compound using a 1/concentration2 weighted linear regression of analyte:internal standard peak area ratio vs. concentration. Calibrators and quality control samples were within 15% of the nominal value for all compounds. Plasma samples with concentrations above the calibration range were diluted into the calibration range with blank plasma prior to reanalysis.

**Bead-based sorting from PBMC**

PBMCs were isolated from heparinized whole blood on Ficoll gradients and cryopreserved for separation in batches. Vials of PBMCs were thawed and counted, and a small aliquot was removed for input analysis prior to bead sorting. CD14 cells were isolated first using the EasySep PE Positive Selection Kit (Stemcell Technologies) according to the manufacturer’s protocols. CD14 PE Clone M5E2 (BD) was used for primary labeling of CD14 cells during the EasySep protocol. Labeled cells were collected and counted. The supernatant fraction containing the CD14-depleted cells was collected and used for CD3 isolation. CD3 cells were isolated using the CD3 MicroBead Kit for non-human primates (Miltenyi Biotec) according to manufacturer’s protocols using MACS LS columns (Miltenyi Biotec). Eluted cells were collected and counted. A portion of each isolated product was stained for purity using CD3 APC Clone SP34-2 (BD), fixed with 10% formalin (Sigma Aldrich) and run on an LSR-2 cytometer using DIVA software (BD). Analysis was performed using FlowJo software version X.0.7 (Treestar). The remainder of cells were pelleted and genomic DNA extracted using MasterPure DNA purification Kit (Epicentre) for PCR analysis.

**Measurement of total SHIV DNA**

Following bead-based sorting and extraction of total genomic DNA, input cellular genome equivalents and total copy number of SHIV DNA were measured by real-time Taqman PCR. SHIV DNA was detected using GAG-specific primers (SIVGAGF: GCA GAG GAG GAA ATT ACC CAG TAC and SIVGAGR: CAA TTT TAC CCA GGC ATT TAA TGT T) and SIVGAG probe (6 FAM- TGT CCA CCT GCC ATT AAG CCC GA -BHQ1) [6]. Cellular genome equivalents were measured using primers (MRPP30F: GAC TTG GAC GTG CGA GCG and MRPP30R: GCC GCT GTC TCC ACA AGT) and probe (MRPP30 Probe: VIC-TCT GAC CTG AAG GCT CTG CGC G-BHQ1) specific for macaque ribonuclease P protein subunit p30 (RPP30) gene. SHIV GAG reactions were spiked with EXO internal control to monitor PCR inhibition [7]. Each 30 μl PCR reaction contained 15 μl of 2x QuantiTec multiplex PCR mix, 900 nM each primer, 250 nM probe, 0.03 units of UNG, and 10 μl of DNA. The cycling conditions were 50°C for 2 minutes, 95°C for 15 minutes, followed by 45 cycles of 94°C for 1 minute and 60°C for 1 minute.

**Supplementary References**

1. Peterson CW, Younan P, Polacino PS, Maurice NJ, Miller HW, Prlic M*, et al.* Robust suppression of env-SHIV viremia in Macaca nemestrina by 3-drug ART is independent of timing of initiation during chronic infection. *Journal of Medical Primatology* 2013; **42(5)**:237-246.

2. Ho O, Larsen K, Polacino P, Li Y, Anderson D, Song R*, et al.* Pathogenic infection of Macaca nemestrina with a CCR5-tropic subtype-C simian-human immunodeficiency virus. *Retrovirology* 2009; **6**:65.

3. Polacino P, Cleveland B, Zhu Y, Kimata JT, Overbaugh J, Anderson D*, et al.* Immunogenicity and protective efficacy of Gag/Pol/Env vaccines derived from temporal isolates of SIVmne against cognate virus challenge. *Journal of Medical Primatology* 2007; **36(4-5)**:254-265.

4. Hu SL, Zarling JM, Chinn J, Travis BM, Moran PA, Sias J*, et al.* Protection of macaques against simian AIDS by immunization with a recombinant vaccinia virus expressing the envelope glycoproteins of simian type D retrovirus. *PNAS* 1989; **86(18)**:7213-7217.

5. Rodrigo AG, Goracke PC, Rowhanian K, Mullins JI. Quantitation of target molecules from polymerase chain reaction-based limiting dilution assays. *AIDS Res Hum Retroviruses* 1997; **13(9)**:737-742.

6. Hofmann-Lehmann R, Swenerton RK, Liska V, Leutenegger CM, Lutz H, McClure HM*, et al.* Sensitive and robust one-tube real-time reverse transcriptase-polymerase chain reaction to quantify SIV RNA load: comparison of one- versus two-enzyme systems. *AIDS Res Hum Retroviruses* 2000; **16(13)**:1247-1257.

7. Limaye AP, Huang M-L, Leisenring W, Stensland L, Corey L, Boeckh M. Cytomegalovirus (CMV) DNA load in plasma for the diagnosis of CMV disease before engraftment in hematopoietic stem-cell transplant recipients. *J Infect Dis* 2001; **183**:377-382.



**Figure S1. Plasma cART concentrations are not perturbed in post-transplant animals.** 1–2 weeks (solid bars) or 13-20 weeks (checkered bars) following initiation of cART in no transplant (blue), SHIV-transplant (red), and transplant-SHIV (green) animals, plasma was collected for analysis of cART concentration by mass spectrometry. (**A**) Components of cART regimen. Plasma concentrations of: (**B**) tenofovir, (**C**) FTC, and (**D**) raltegravir.

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**Figure S2. Peripheral blood monocyte:CD4**+ **T-cell ratios are elevated in post-transplant animals prior to and following SHIV challenge.** Monocyte and CD4+ T-cell counts in peripheral blood were measured and compared longitudinally in no transplant (blue), SHIV-transplant (red), and transplant-SHIV (green) animals. Arrows indicate time of cART initiation and autologous transplant, where applicable, for each group. (**A**) Monocyte counts/ µl over the course of SHIV infection. (**B**) Average monocyte counts over 4–6 weeks prior to SHIV challenge. (**C**) Average monocyte counts over 9–10 weeks post-challenge, prior to Group III animals initiating cART. (**D**) Ratios of monocytes/µl to CD4+ T-cells/µl over the course of SHIV infection. ( **E**) Average monocyte:CD4+ T-cell ratios over 4–6 weeks prior to SHIV challenge. (**F**) Average monocyte:CD4+ T-cell ratios over 9–10 weeks post-challenge, prior to Group III animals initiating cART. Error bars represent standard deviations between weekly measurements. Statistical significance between collective measurements from Groups I-II and Group III was measured by single-tailed Student’s t-test. \*\* represents *p* < 0.01, and \*\*\*\* represents *p* < 0.0001.



**Figure S3. Total SHIV DNA in CD14+ myeloid cells from post-transplant animals is comparable to control animals.** PBMC were collected longitudinally from no transplant (blue), SHIV-transplant (red), and transplant-SHIV (green) animals for sequential CD14+ and CD3+ magnetic bead-based sorting. (**A**) CD14+ purity and CD3+ contamination were measured by flow cytometry following sorting with anti-CD14 beads. Total DNA was isolated following CD14+ and subsequent CD3+ bead sorting. Taqman was used to measure total SHIV DNA normalized to genomic DNA in (**B**) CD14+ and (**C**) CD3+ cells. (**D**) Ratio of genomic DNA-normalized total SHIV DNA in CD14+ vs. CD3+ populations. Triangles indicate increasing time post-SHIV challenge.



**Figure S4.** **Comparison of T-cell subset homeostasis in peripheral blood and gastrointestinal tract between post-transplantand control animals.** T-cell subset percentages were measured by flow cytometry from PBMC or dissociated gastrointestinal (GI) biopsy samples collected from no transplant (blue), SHIV-transplant (red), and transplant-SHIV (green) animals. (**A**) CD4+CCR5+, (**B**) CD4+ naïve, (**C**) CD8+ naïve, (**D**) CD4+central memory, (**E**) CD8+central memory, (**F**) CD4+effector memory, and (**G**) CD8+effector memory populations from peripheral blood are shown. Phenotypic markers: Naïve: CCR7+CD45RA+CD28+CD95-; Central Memory: CCR7+CD45RA‑; Effector Memory: CCR7-CD45RA-. (**H**) Upper GI CD3+CD4+, (**I**) Upper GI CD3+CD4+CCR5+, (**J**) Lower GI CD3+CD4+, (**K**) Lower GI CD3+CD4+CCR5+. Green and black arrows denote cART initiation for transplant-SHIV and SHIV-transplant/No transplant animals, respectively. CBC measurements were used to calculate absolute cell counts from peripheral blood.

**Supplementary Table 1.** Summary of SHIV drug resistance mutations in seven pigtailed macaques

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Reverse Transcriptase Mutations | | | Integrase Mutations | | | |
| Animal ID | Group | K56R | K70E | M184V | E92Q | Y143R/H/C | Q148H/K/R | N155H |
| Z09087 | No transplant | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 |
| Z09106 | No transplant | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 |
| Z09192\*\* | No transplant | 0/4 | 0/4 | 0/4 | 0/2 | 0/2 | 0/2 | 0/2 |
| Z09144 | SHIV-transplant | 0/12 | 0/12 | 0/12 | 0/12 | 0/12 | 0/12 | 0/12 |
| Z08214 | SHIV-transplant | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 |
| A11202 | Transplant-SHIV | 5/9 | 3/9 | 1/9 | 0/10 | 0/10 | 0/10 | 6/10 |
| A11205 | Transplant-SHIV | 0/7 | 0/7 | 0/7 | 0/8 | 0/8 | 0/8 | 2/8 |

\*Data represent the proportion of measurements made over the first 6–7 months of SHIV infection in the indicated animal that displayed >2% mutation frequency at the indicated site

\*\*RT mutations were measured at weeks 5,7,17 and 25. Integrase mutations were measured at weeks 17 and 25.

**Supplementary Table 2.** Primer sequences used to amplify the pol and env regions of the genome

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| --- | --- |
| **Genome region** | **Primer sequence** |
| pol1 1757F-out | GCC CCA AGA AGA CAG GGA TGC |
| pol1 3552R-out | CCT GCC AAT AGT CTG TCC ACC A |
| pol2 3218F-out | GGA CTG AGA TGG CAG AAG CA |
| pol2 4976R-out | ACC ATG CCC ATC CGA CCT TA |
| env1 5972F-out | AGA CTA GAG CCC TGG AAG CA |
| env1 7882R-out | GCT CCC AAG AAC CCA AGG AA |
| env2 7249F-out | GCA TAG GAC CAG GAC AAG CA |
| env2 9353R-out | TCC TCA TCC TCC TGT GCC TC |