OMTM, Volume *25*

Supplemental information

Pre-clinical data supporting immunotherapy for HIV using CMV-HIV-specific CAR T cells with CMV vaccine

Min Guan, Laura Lim, Leo Holguin, Tianxu Han, Vibhuti Vyas, Ryan Urak, Aaron Miller, Diana L. Browning, Liliana Echavarria, Shasha Li, Shirley Li, Wen-Chung Chang, Tristan Scott, Paul Yazaki, Kevin V. Morris, Angelo A. Cardoso, M. Suzette Blanchard, Virginia Le Verche, Stephen J. Forman, John A. Zaia, John C. Burnett, and Xiuli Wang

Supplemental Data

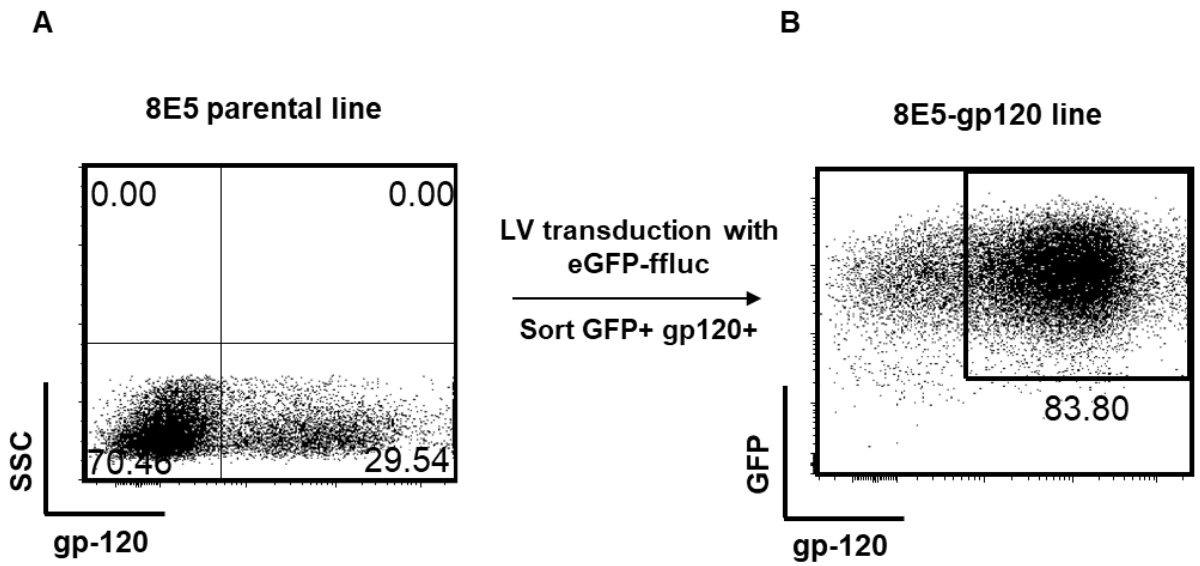

Fig. S1. Development of 8E5-gp120 cell line.

Parental 8E5 cells derive from HIV-infected lymphoblastic cells and carry a single, reverse transcriptase (RT)-defective copy of an integrated HIV genome. **(A)** Flow cytometry analysis using anti-gp120 monoclonal antibody staining showed ~30% of the parental cells express gp120. **(B)** Cells were transduced with a lentiviral vector encoding eGFP and firefly luciferase (ffLuc) and then eGFP and gp120 double-positive cells were sorted and expanded for in vitro experiments.

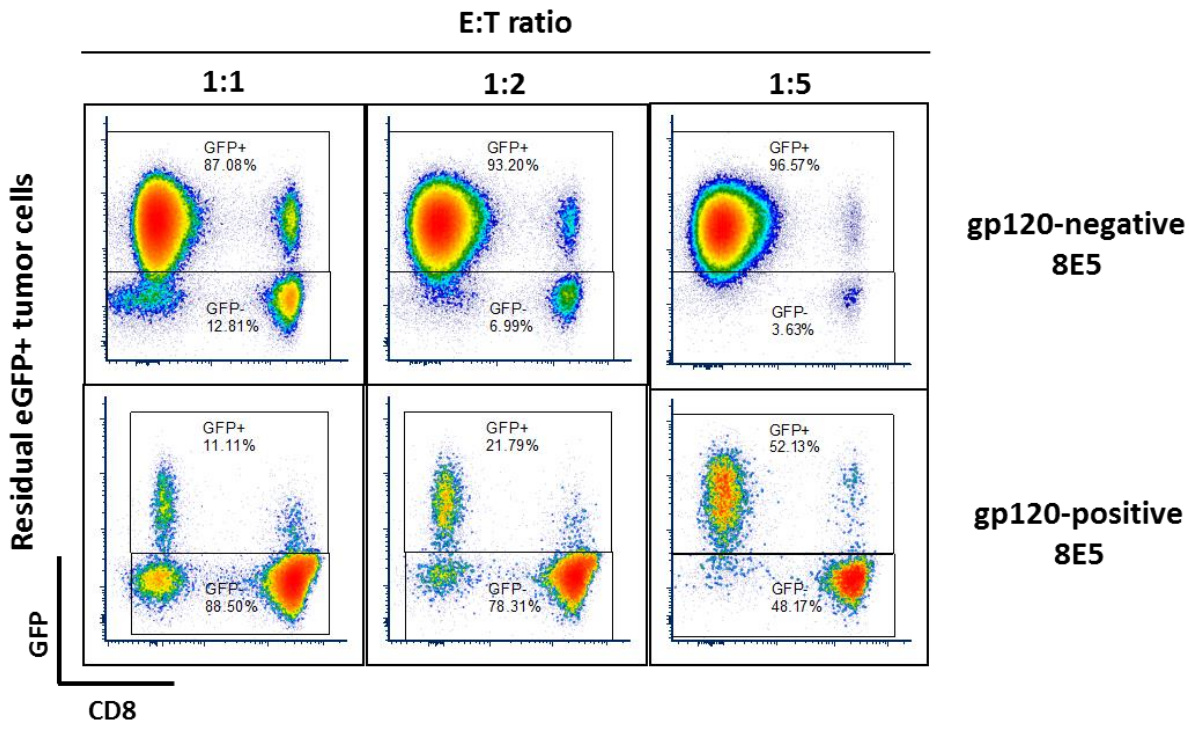

Fig. S2. Specific cytotoxicity of N6-CAR T cells against gp120-positive cells.

N6-CAR T cells derived from an HIV^{neg} donor were co-cultured at various E:T ratios (1:1, 1:2 or 1:5) with eGPF+ 8E5 cells sorted for gp120 expression. Residual eGFP + tumor cells were measured by flow cytometry after 96 hours.

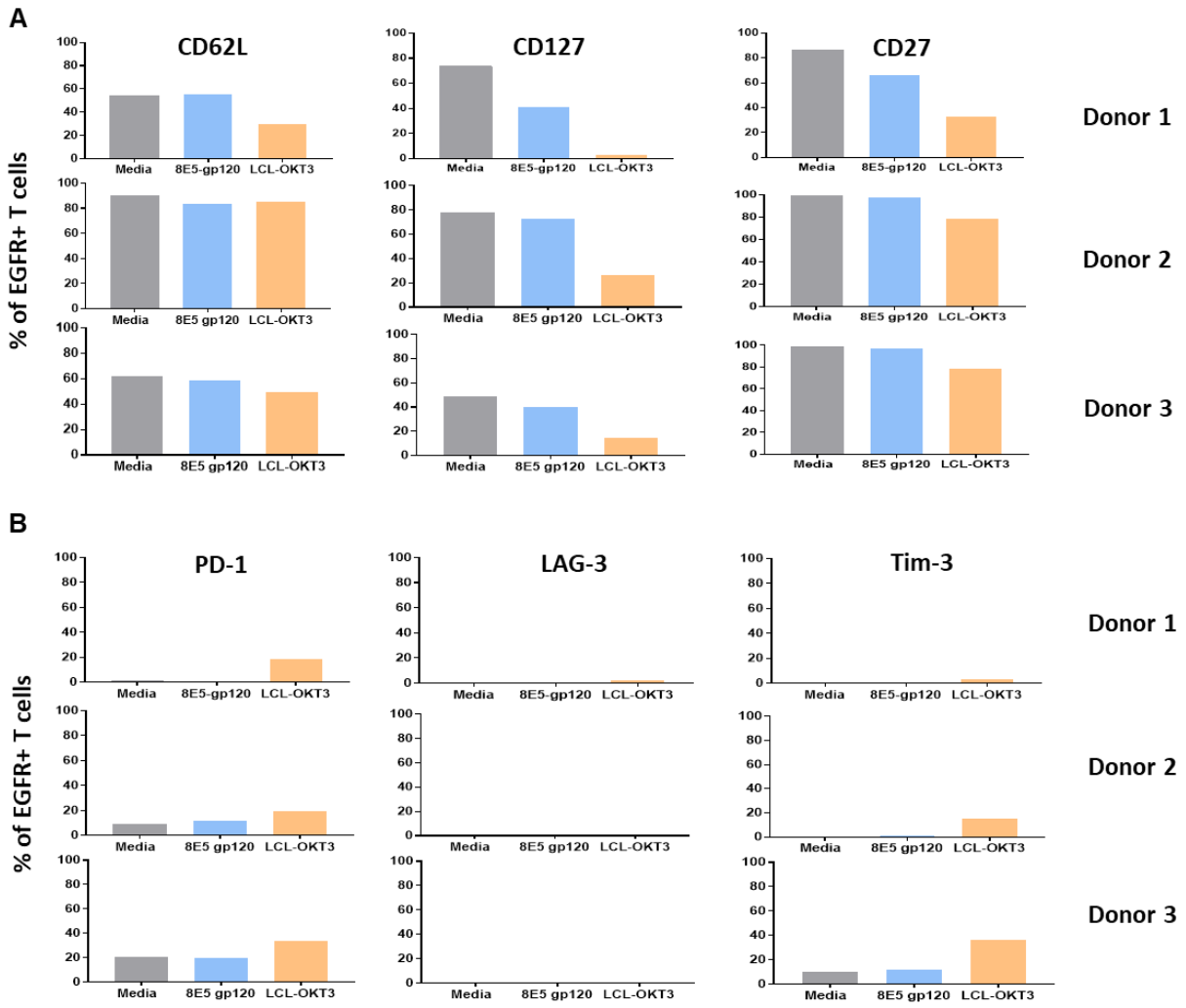

Fig. S3. Phenotypic characterization of N6-CAR T cells after stimulation with 8E5-gp120 cells.

N6-CAR T cells derived from three HIV^{neg} donors were cocultured at an E:T ratio of 1:1 with either 8E5-gp120 cells, LCL-OKT3 cells, or medium for 96 hours before flow cytometric analysis of the expression of (**A**) memory (CD62L, CD127 and CD27)**,** or (**B**) exhaustion (LAG-3, PD-1 and Tim-3) markers.

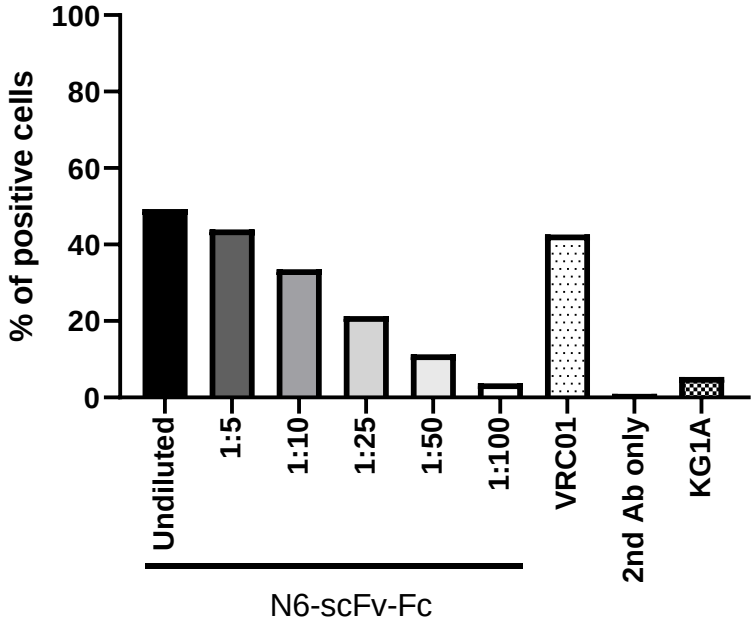

Fig. S4. Specific binding of N6 scFv-Fc on gp120-expressing cells.

8E5-gp120 cells were stained with soluble N6 scFv-Fc at the indicated dilutions. Positive cells were quantified by flow cytometry. Staining with the anti-gp120 bNAb VRC01 was used as positive control, and gp120-negative KG-1a cells or staining with the secondary antibody alone were used as negative controls.

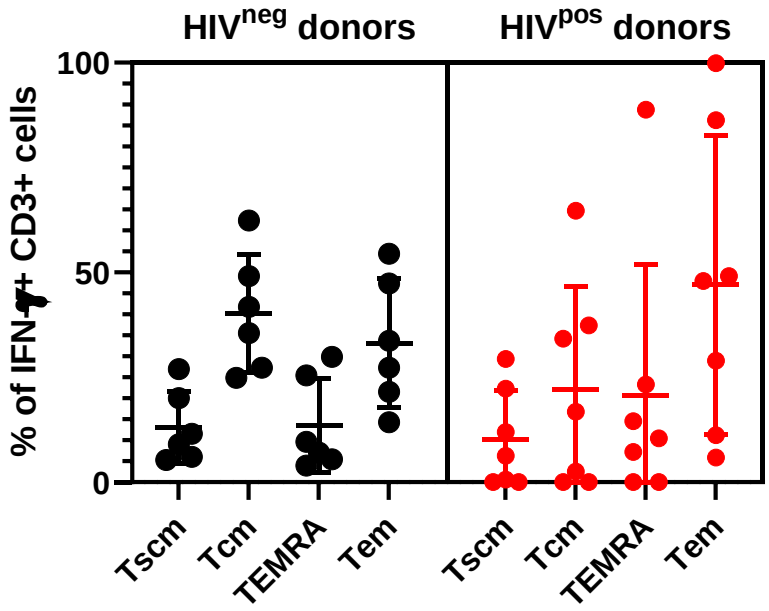

Fig. S5. Memory cell subsets in CMV-specific T cells isolated from HIV^{neg} and HIV^{pos} donors.

CMV-specific T cells (IFN-γ+CD3+) isolated from HIV^{neg} and HIV^{pos} donors were enriched using the CliniMACS Prodigy system and immunostained with anti-CD27 and anti-CD45RA antibodies. Flow cytometric analysis show the percentage of CMV-specific T cells that are CD27+CD45RA+ stem cell memory T cells (Tscm), CD27+CD45RA- central memory (Tcm), CD27-CD45RA+ effector memory RA (TEMRA), and CD27-CD45RA- effector memory T cells (Tem). Lines indicate means ± SD; n = 5 donors per group.

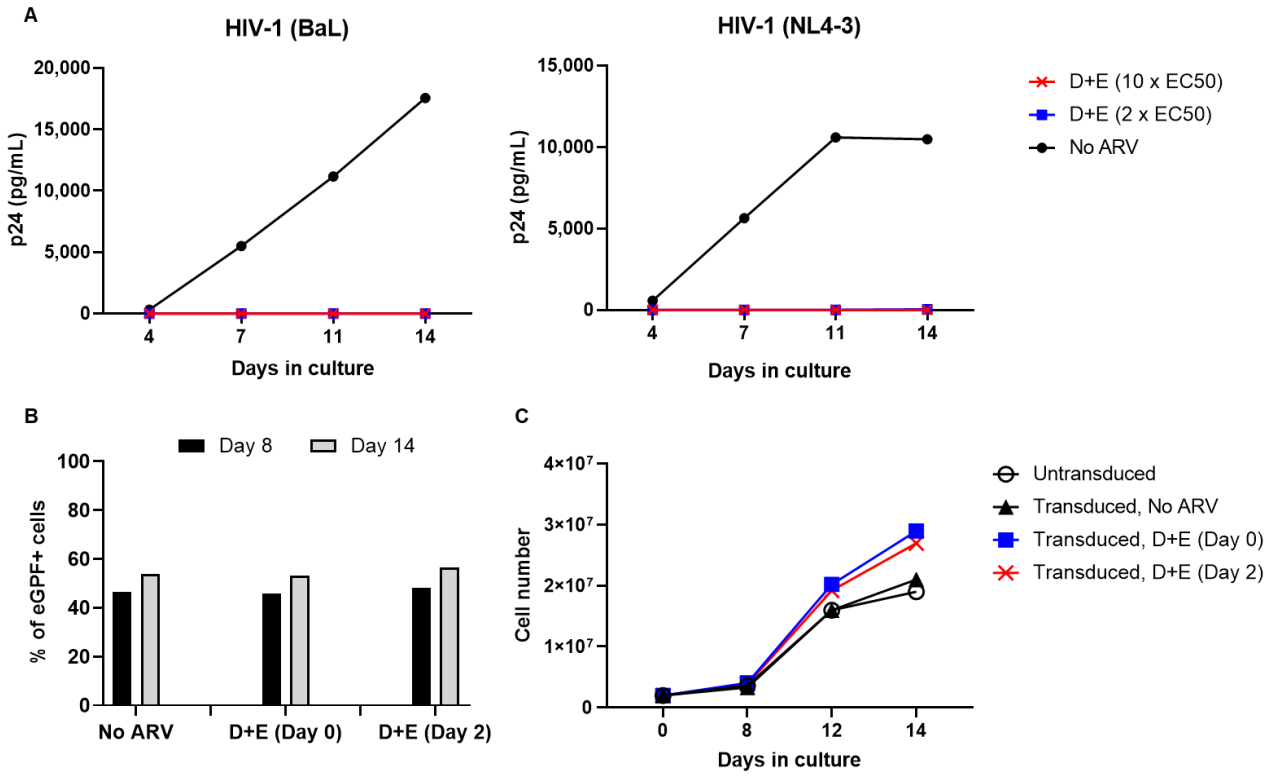

Fig. S6. *In vitro* HIV replication, lentiviral transduction and cell expansion in presence of antiretroviral drugs (ARV).

(**A**) The HIV protease inhibitor darunavir (D, EC_{50} = 4.3 nM) and the HIV fusion inhibitor enfurvitide (E, EC_{50} = 27.9 nM) prevent HIV replication when added in $CD8^+$-depleted PBMCs infected with HIV-1 BaL (left panel) or HIV-1 NL4-3 (right panel) strains. (**B**) Darunavir (43 nM) and enfurvitide (279 nM) were added on the day of (Day 0) or 2 days after (Day 2) transduction of PBMCs with a lentiviral vector expressing eGFP. Flow cytometric analysis of eGFP expression 8 and 14 days after lentiviral transduction is shown. (**C**) Cell expansion of untransduced or transduced PBMCs in the presence or absence of antiretroviral drugs.

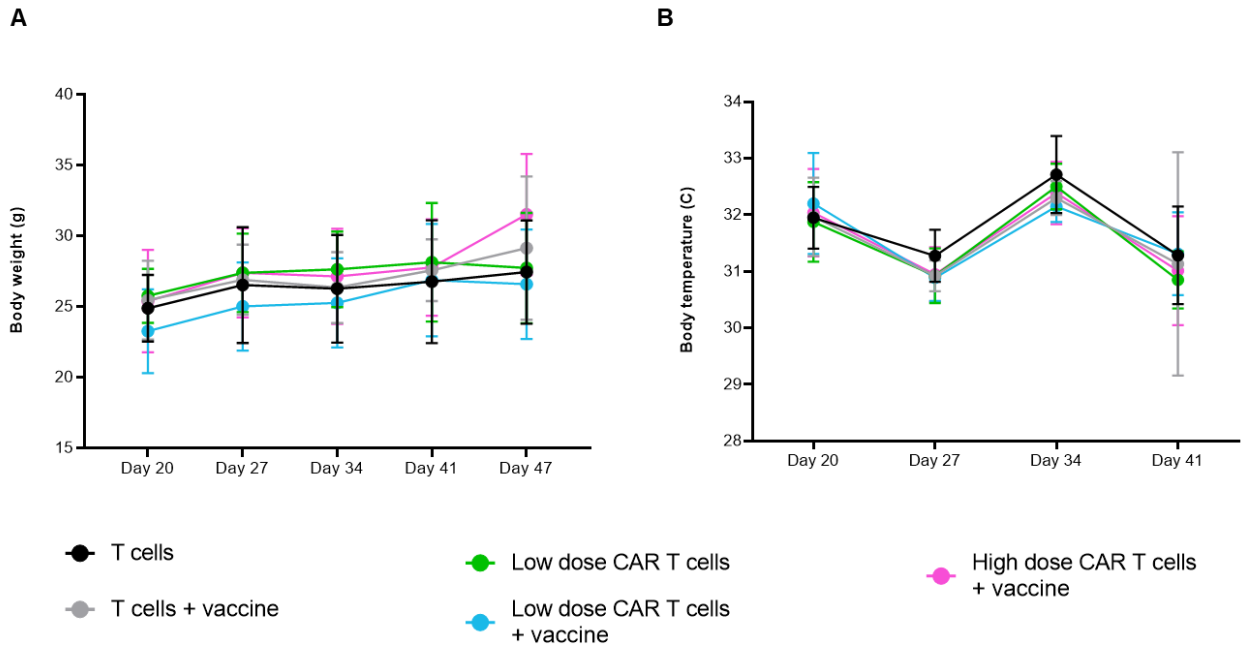

Fig. S7. Body weight and temperature in HIV-infected hu-PBMC-NSG mice treated with ART, CMV-HIV CAR T cells, with or without CMVpp65 vaccine.

Body weight (**A**) and temperature (**B**) were monitored weekly in the hu-PBMC mouse model upon transplant with HIV^{neg} donor-derived PBMCs (Day 0). Mice started oral ART regimen on Day 12, received a single IV dose of CMV-HIV CAR T cells (low [0.1×10^6] or high [1×10^6] dose) on Day 21 and CMVpp65 vaccine on Day 28. No statistical significance between the groups was observed using ANOVA mixed-effects analysis. Groups are as follows: T (CMV-negative) cells, n=8; T cells (CMV-negative) + vaccine, n=8; Low dose CAR T cells, n=8; Low dose CAR T cells + vaccine, n=8; High dose CAR T cells + vaccine, n=9.

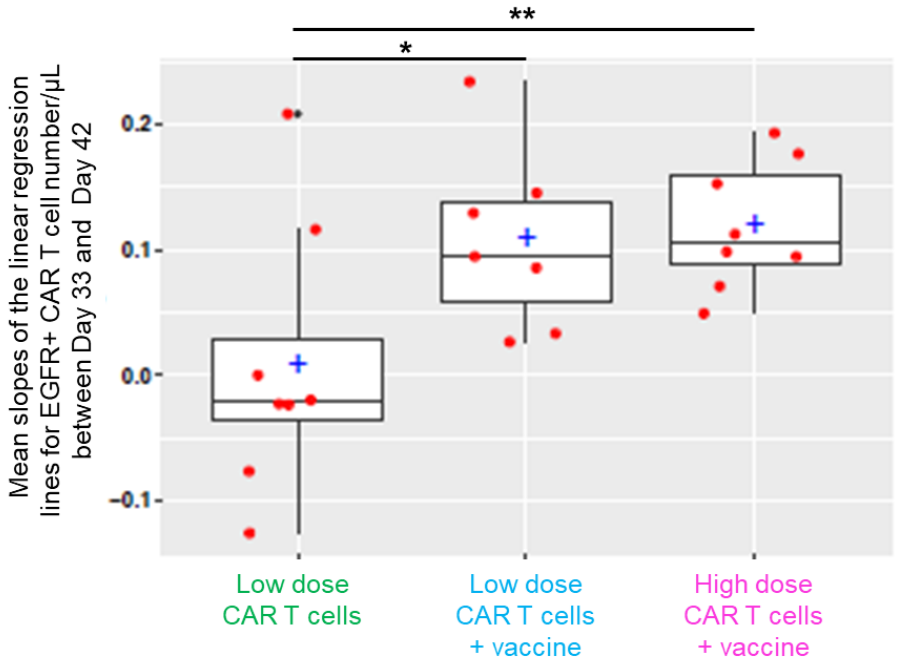

Fig. S8. EGFR+ CAR T cell expansion in the peripheral blood between Day 33 and Day 42 in HIV-infected hu-PBMC-NSG mice treated with ART, CMV-HIV CAR T cells, with or without CMVpp65 vaccine.

EGFR+ CAR T cell expansion in the peripheral blood was assessed based on the mean slopes of the linear regression lines for EGFR+ CAR T cell number/µL using a log10 transformation from Day 33 and Day 42. Statistical significance was determined using one-sided Tukey contrasts *P-value = 0.03; **P-value = 0.02. n=8/group, same groups as in Fig. 6C.

Table S1. HIV^{pos} donor information.

Donor ID	Gender	Age	Ethnicity	Race	ART Regimen
HIV#551	N/A	N/A	N/A	N/A	N/A
HIV#552	Male	52	Non-Hispanic	White	Genvoya® (elvitegravir, cobicistat, emtricitabine, and tenofovir alafenamide)
HIV#553	Female	54	Non-Hispanic	African American	Genvoya® (elvitegravir, cobicistat, emtricitabine, and tenofovir alafenamide)
HIV#572	Female	50	Hispanic	White	Biktarvy® (bictegravir, emtricitabine and tenofovir alafenamide)
HIV#573	Male	54	Non-Hispanic	White	Atripla® (efavirenz, emtricitabine, and tenofovir)
IEQR#2	Male	54	Non-Hispanic	Caucasian	Descovy, Sustiva
IEQR#3	Male	54	Non-Hispanic	Caucasian	Descovy, Sustiva

N/A: information not available

Supplemental Methods

Antibodies

Fluorochrome-conjugated isotype controls against CD3 (#563109), CD4 (#557582), CD8 (#348793), IFN-γ (#554701), CD27 (#555440), CD45RA (#550855), CD62L (#341012), CD127 (#560822), programmed cell death-1 (PD-1) (#551892), lymphocyte-activation gene-3 (LAG-3, #565720) and T cell immunoglobulin and mucin domain-3 (Tim-3, #563422) were obtained from BD Biosciences (San Diego, CA). The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: anti-HIV-1 gp120 Monoclonal (VRC01) from Dr. John Mascola (cat# 12033). Biotinylated anti-EGFR antibody Erbitux® (cetuximab) was obtained from the City of Hope pharmacy. Antibody against EGFR was obtained from eBioscience (San Diego, CA). CellTrace™ Violet dye (CTV) was purchased from Invitrogen (Carlsbad, CA). All monoclonal antibodies and CTV were used according to the manufacturer's instructions.

Reagents

CliniMACS Prodigy® TS500 tubing sets, MACS GMP PepTivator® HCMV pp65, CCS Reagent, CliniMACS PBS/EDTA buffer and TexMACS™ GMP medium were all purchased from Miltenyi Biotec. CliniMACS PBS/EDTA with 2.5% human serum albumin (HSA; Grifols Therapeutics, Los Angeles, CA) was used as the elution buffer. GMP-grade cell transfer bags and luer/spike adaptors were purchased from BD Medical (Franklin Lakes, NJ). pepMix HCMVA (pp65; pp65pepmix) was purchased from JPT Peptide Technologies. GmbH was used for pulsation on PBMCs according to the manufacturer's instructions. Antiretroviral drug darunavir was obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH (Cat# 11447) from Tibotec, Inc and enfuvirtide (Fuzeon, Genentech) were reconstituted in water.

Synthesis of scFv-Fc of N6

The anti-gp120 N6 monoclonal antibody (mAb) variable domains were reformatted into a recombinant single-chain scFv-Fc antibody fragment. The cDNA encoding the N6 variable light and heavy chain domains (in VL-linker-VH- orientation) were synthesized with a (Gly4Ser)3 linker and fused to an IgG4 Fc domain. Briefly, the scFv-Fc of N6 was cloned into the Lonza pEE12.4 vector and transiently transfected using the EXPI293 expression system. The culture was then clarified by centrifugation (1,000 × g, 5 min), followed by 0.22 µm sterile filtration. The clarified harvest was treated overnight with AG 1-X8 strong anion exchange resin and affinity purified by protein A chromatography (ProSep vA high-capacity resin, EMD Millipore). Pooled eluates containing N6 scFv-Fc (V_L-V_H) were dialyzed using a Slide-A-Lyzer 20k MWCO cassette vs. PBS buffer. The final dialyzed sample was sterile filtered using 0.22 µm PES filter membrane and stored at 4°C. The test reagent was assayed for expression by SDS-PAGE and ELISA assays.

Tissue cross-reactivity analysis

Charles River Laboratories, Inc. performed the cross-reactivity study of N6 scFv-Fc. First, N6 scFv-Fc was tested for specific reaction on positive control (gp160-transfected HEK293T cells expressing gp120) and negative control parental HEK293T cells (gp120-negative) at 5 µg/mL and 15 µg/mL. The test article was substituted with a human IgG4κ antibody, designated HuIgG4 (control article) and other controls were produced by omission of the test or control articles from the assay (assay control). The tissue panel used as the test system for the in vitro cross-reactivity study includes all the tissues recommended in the FDA, Center for Biologics Evaluation and Research (CBER) document *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use*. Fresh unfixed tissues were collected as surgical or autopsy specimens from humans and frozen in Tissue-Tek® OCT at -85-70°C. Sections were cut at approximately 5 µm and fixed in acetone for 10 min at room temperature. Just prior to staining, the slides were fixed in 10% neutral-buffered formalin (NBF) for 10 seconds at room temperature. The labeled secondary antibody was allowed to attach specifically to the unlabeled primary antibody (either test or

control article at 5 µg/mL and 15 µg/mL) by overnight incubation of the primary/secondary antibody mixtures. The test or control article was mixed with biotinylated F(ab')2 donkey anti-human IgG, Fcγ fragment-specific (DkαHuIgG) antibody at concentrations which achieved a primary:secondary antibody ratio of 1:1.5. Precomplexed antibodies were incubated overnight at 2 to 8°C. Prior to use of the antibody on the subsequent day, human gamma globulins were added to each vial to achieve a final concentration of either 4.5 mg/mL (higher concentration of secondary antibody) or 1.5 mg/mL (lower concentration of secondary antibody), and antibodies were incubated for at least 2 hours at 2 to 8°C. On the day of staining, the slides were rinsed twice with Tris-buffered saline, 0.15M NaCl, pH 7.6 (TBS). Next, the slides were incubated with the avidin solution for 15 min, rinsed once with TBS, incubated with the biotin solution for 15 min, and rinsed once with TBS. The slides were then treated for 20 min with a protein block (TBS + 1% bovine serum albumin (BSA); 0.5% casein; and 1.5% normal donkey serum) designed to reduce nonspecific binding. Following the protein block, the precomplexed primary and secondary antibodies were applied to the slides for 2 hours. Next, the slides were rinsed twice with TBS, and endogenous peroxidase was then quenched by incubation of the slides with the Dako peroxidase blocking reagent for 5 min. Next, the slides were rinsed twice with TBS, treated with the ABC Elite reagent for 30 min, rinsed twice with TBS, and then treated with DAB for 4 min as a substrate for the peroxidase reaction. All slides were rinsed with tap water, counterstained, dehydrated, and mounted. TBS + 1% BSA served as the diluent for all antibodies and ABC reagent. Separate cryosections from each human test tissue were stained in parallel for the expression of human β2-microglobulin (a relatively ubiquitous epitope) using a polyclonal rabbit antibody directed against human β2-microglobulin. All evaluated human test tissues stained positive for β2-microglobulin, indicating their suitability in the cross-reactivity evaluation. After staining, slides were visualized and evaluated under light microscopy by a pathologist.

Flow cytometry

Cells were stained with optimized antibody panels for 20 min at 4°C followed by two washes with PBS. Data acquisition for all experiments involving flow cytometry was performed on a MACSquant (Miltenyi Biotec) and analyzed using FCS Express V7 (De Novo Software, Glendale, CA).

Peripheral blood samples were collected by retro-orbital bleeding under general anesthesia and stained for 30 min with BV711-conjugated anti-human CD3, APC-conjugated anti-human CD4, BB515-conjugated anti-human CD8, BUV395-conjugated anti-human CD45 (BD Biosciences, San Jose, CA), and BV421-conjugated anti-human EGFR (Biolegend, San Diego, CA). Stained peripheral blood samples were then lysed with red blood cell lysis buffer and absolute cell counts calculated using BD Liquid Counting Beads (BD Biosciences, San Jose, CA). Flow cytometry was performed using BD Fortessa II instrument (BD Biosciences) and analyzed with FlowJo software (BD formerly TreeStar).

Tissue samples were collected at necropsy and processed immediately for cell isolation and flow cytometry analysis. Bone marrow mononuclear cell suspensions were first stained with amine binding dye for dead cell exclusion (Biolegend) and then stained with anti-human CD3 (BD clone UCHT1), EGFR (Miltenyi biotinylated clone REA688), CD4 (Biolegend clone RPA-T4), anti-human CD8 (BD clone RPA-T8), CD62L (Biolegend clone DREG-56), and CD27 (Biolegend clone M-T271) in brilliant staining buffer (BD) containing 0.5% human serum albumen and 0.5% gamma globulin. Primary EGFR antibody staining was finished with a streptavidin conjugate (eBioscience) and fixed in 4% PFA. Samples were acquired the next day on a BD Fortessa SORP cytometer. Data was analyzed using FlowJo Software (BD formerly TreeStar). Cell doublets and dead cells were excluded prior to evaluation of the T cell lineage and phenotypic markers.

Intracellular HIV p24 staining

Samples of peripheral blood and single cell suspensions of mouse bone marrow (femurs +/- tibias) were collected at time of euthanasia. Single cell suspensions were made following previously established protocols.[1] Briefly for bone marrow cells, femurs and tibia were dissected and collected from euthanized mice and placed in ice cold PBS. Bones were cleaned thoroughly to remove all connective and muscle tissue, then using a scalpel blade the heads of the bones were removed. Bones were placed in 0.5 mL microcentrifuge tube with a premade hole by using a 20G needle. Bones were placed cut surface down and

a 0.5 mL tube was placed in a 1.5 mL microcentrifuge tube and centrifuged at > 10,000 × g for 15 sec. Cell pellet was resuspended in ACK lysis buffer incubated for 5 min and washed with PBS. Cells were resuspended in PBS + 2% FBS and then processed for FACS staining or frozen in 10% Cryostor (Stem Cell Technologies, Vancouver, BC). For intracellular staining, BD Cytofix/Cytoperm™ kit (BD Biosciences, San Jose, CA) was used following manufacture's protocol. After surface markers staining (CD45, CD3, CD4, CD8, EGFR), cells were permeabilized and intracellular staining of KC57-FITC monoclonal antibody Fortessa II instrument (BD Biosciences) and analyzed with FlowJo software (BD formerly TreeStar).

ELISA assay

Quantification of HIV-1 p24 was measured on the supernatants as per the manufacturer's instructions (Alliance ELISA; Perkin-Elmer Life Sciences, Boston, MA) with the assay's Lower Limit of Quantification (LLOQ) being 12.5 pg/mL.

Plasma HIV qRT-PCR

Plasma viremia was assayed using one-step reverse transcriptase real-time PCR [TaqMan assay] with automated CFX96 TouchTM Real Time PCR Detection System (Bio-Rad). qPCR primer sets were taken from previously published studies.[2] HIV-1 level in peripheral blood was determined by extracting RNA from blood plasma using the QIAamp Viral RNA mini kit (Qiagen) and performing Taqman qPCR using either a primer and probe set targeting the HIV-1 LTR region [FPrimer: GCCTCAATAAAGCTTGCCTTGA, RPrimer: GGCGCCACTGCTAGAGATTTT, Probe: 5'FAM/AAGTAGTGTGTGCCCGTCTGTTGTGTGACT /3IABkFQ] or the HIV-1 Pol region [FPrimer: GACTGTAGTCCAGGAATATG, RPrimer: TGTTTCCTGCCC TGTCTC, Probe: 5'Cy5/CTTGGTAGCAGTTCATGTAGCCAG/3'IABkFQ], using the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems). According to the manufacturer's instruction (QIAamp Viral RNA mini kit [Qiagen]), the protocol is designed for purification of viral RNA from minimal 140 µL plasma. In a standard Taqman qPCR-based HIV-1 plasm viral load test, the limit of detection (LOD) is typically about 40 copies/mL when viral RNA isolated from 140 µL of plasma sample is applied. In our animal study, the plasma sample was expanded by dilution (generally 1 to 3 dilution) because only limited volume of plasma (20 - 40 µL) was available. The LOD of the diluted samples was around ~2,000 RNA copies/mL using the HIV LTR primer and ~500 RNA copies/mL using the HIV Pol primer under our experimental condition. Therefore, we defined that the value below those LOD numbers is undetectable.

Supplemental References

1. Au - Amend, S.R., Au - Valkenburg, K.C., and Au - Pienta, K.J. (2016). Murine Hind Limb Long Bone Dissection and Bone Marrow Isolation. JoVE, e53936. doi:10.3791/53936.
2. Satheesan, S., Li, H., Burnett, J.C., Takahashi, M., Li, S., Wu, S.X., Synold, T.W., Rossi, J.J., and Zhou, J. (2018). HIV Replication and Latency in a Humanized NSG Mouse Model during Suppressive Oral Combinational Antiretroviral Therapy. J Virol *92*. 10.1128/JVI.02118-17.