**SUPPLEMENT**

Mechanisms of residual immune activation in HIV-1 infected human lymphoid tissue *ex vivo*.

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**MATERIALS AND METHODS**

**Sample preparation**

Tonsils were sectioned into 2x2x2mm blocks and cultured at the air-liquid interface on collagen sponges. Nine blocks are placed on each sponge in 6 well plates with 3ml culture medium consisting of RPMI 1640 with L-glutamine, 15% FBS, 100M MEM-nonessential amino acids, 1mM MEM sodium pyruvate, 50g/ml gentamicin, and 2.5g/ml amphotericin B (all Thermo Fisher Scientific, Waltham, MA) and 15% fetal bovine serum (Gemini Bioproducts, Sacramento, CA).

Previous work by our lab and others has demonstrated retention of T cells, B cells, macrophages, and follicular dendritic cell networks in *ex vivo* lymphoid tissue cultures when cultured for two weeks, as well as the ability of viruses and small compounds to penetrate tissue blocks [1-5].

**Evaluation of HIV-1 replication**

HIV-1 replication was measured by a dynamic immunofluorescent cytometric bead assay of HIV-1 p24gag antigen in tissue culture supernatants. Briefly, samples were lysed with 1% Triton X-100 final and incubated at 37°C for one hour. Samples and standards were incubated with anti-p24 antibody coupled MagPlex beads (Luminex, Austin, TX) and incubated for two hours at room temperature (RT). Plates were washed on a magnetic plate washer (Biotek, Winooski, VT). RD1-labeled anti-p24 KC57 antibody (Beckman Coulter, Miami, FL) was added and plates were incubated for one hour at RT. Plates were washed and PBS was added to all wells. Analysis was performed on a Luminex 200 acquiring 100 beads/well.

**Evaluation of endogenous herpesviruses replication**

**Qiagen DNA extraction**

For each condition (uninfected, HIV-1-infected, HIV-1 + RTV) and for three time points (day 0, 6 and 12), nine tissue blocks were collected and stored in RNAlater (Thermo Fisher) at -20°C to stabilize and protect cellular nucleic acids. DNA was isolated from the tissue using QIAamp DNA Mini kit (Qiagen, Germantown, MD) according to manufacturer guidelines. Briefly, tissues were enzymatically lysed, ethanol was added, and lysates were loaded onto two different spin columns. Wash buffers were used to purify the DNA which was then eluted off the columns into sterile DNase/RNase free 1.5ml microcentrifuge tube with a final volume of 200μl.

**Droplet digital PCR (ddPCR)**

Droplet digital PCR (BioRad, Hercules, CA) was used to quantify DNA copies of different human herpesviruses (HHVs): herpes simplex virus 2 (HSV-2), HHV-3, HHV-4 (or EBV), HHV-5 (or CMV), HHV-6 and HHV-7. Ribonuclease P protein subunit p30 (RPP30) was used as a housekeeping gene to normalize DNA levels. The primer-probes were conjugated with HEX (6-carboxy-2,4,4,5,7,7 hexachlorofluorescein succinimidyl ester) and BHQ1 (black hole quencher 1) or with FAM (6-carboxyfluorescein) and TAM (tetramethylrhodamine quencher) (Sigma-Aldrich, Saint Louis, MO), see Supplemental Table 1 for primer-probe specifics.

For each single reaction, a total of 11μl of 2x ddPCR supermix for probes (Bio-Rad), 1.1 μl of each 20× primer-probe mix, 5μl of DNA template and 5μl of DEPC water was used. Next, a droplet generator, which mixed 20μl of the reaction with 20μl of droplet generation oil, allowed the formation of droplets. PCR amplification was performed into a 2720 Thermal Cycler (Applied Biosystems/Thermo Fisher) with the following settings: 94°C for 10 minutes, 40 cycles of 94°C per 10 minutes, 57.1°C for 1 minute (for HHV-3, HHV-4, CMB UL-55, and HHV-6 primers/probes) or 59°C (for RPP-30, HSV-2, AND HHV-7 primers/probes), followed by 98°C per 10 minutes. The droplet reader was used to count positive and negative generated droplets. Data obtained were analysed by QuantaSoftTM Analysis Pro (version 1.0.596) software.

**PRIMER/PROBE SEQUENCES**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Forward sequence** | **Reverse sequence** | **Probe Sequence** |
| **RPP30-HEX** | GATTTGGACCTGCGAGCG | GCGGCTGTCTCCACAAGT | TTCTGACCTGAAGGCTCTGCGC |
| **HSV-2 UL27-FAM** | CGCATCAAGACCACCTCCTC | GCTCGCACCACGCGA | CGGCGATGCGCCCCAG |
| **HHV-3 ORF62-FAM** | CGGCATGGCCCGTCTAT | TCGCGTGCTGCGGC | ATTCAGCAATGGAAACACACGACGC |
| **HHV-4 LMP2A-FAM** | AACGATGAGGAACGTGAAT | AGTCATCCCGTGGAGAGTA | AGAGCCCCCACCGCCTTA |
| **CMV UL55-HEX** | TGGGCGAGGACAACGAA | TGAGGCTGGGAAGCTGACAT | TGGGCAACCACCGCACTGAGG |
| **HHV-6 U67-FAM** | CGCTAGGTTGAGGATGATCGA | CAAAGCCAAATTATCCAGAGCG | CCCGAAGGAATAACGCTC |
| **HHV-7 U67-FAM** | AGCGGTACCTGTAAAATCATCCA | AACAGAAACGCCACCTCGAT | GAGAACATCGCTCTAACTGGATCA |

**Supplemental Table 1:** Forward and reverse primers, and probes for detection by ddPCR of RPP30, HSV-2, HHV-3, HHV-4, CMV (HHV-5), HHV-6, HHV-7. RPP-30 and CMV probes were conjugated with HEX, all the other probes with FAM.

**CMV infectivity assays and block transfer**

Tissue blocks from uninfected, HIV-1-infected, and HIV-1-infected and RTV treated conditions were collected and frozen at day 12. MRC5 cells (human lung fibroblasts, ATCC, Manassas, VA) were plated into 24 well plates at 6x104 cells/well. The next day, tissues blocks were thawed and immediately physically dissociated in 500l PBS using pestles. Homogenized tissue was centrifuged at 2000 x g for 5 minutes and supernatant was collected and applied to MRC5 cells for four hours at 1:10 dilutions in DMEM, then removed and 1ml overlays of 2% carboxymethylcellulose (CMC) in DMEM were added to each well. Cultures were stopped at day 13, overlays were removed, cells were washed, fixed with 10% formalin, stained with crystal violet and cells were microscopically examined.

**EV isolation and characterization**

Supernatants were collected from experimental conditions (uninfected, uninfected and treated with RTV or NVP, HIV-1-infected, and HIV-1-infected and treated with RTV or NVP) at day 9 from multiple tissue donors and centrifuged at 2,000 *g* for 10 minutes and stored at -80°C. Supes were thawed, pooled, 0.45m filtered, and EVs were isolated by different procedures.

EVs from HIV-1-infected + RTV or NVP experiments were isolated from culture supernatants using membrane affinity spin columns (Qiagen exoEASY Maxi kits) according to manufacturer guidelines, followed by buffer exchange and concentration in 100K MWCO centrifugal filters.

To completely remove any HIV-1 virions from EV preparations, EVs were isolated using iodixonal gradients, as previously published [6]. Briefly, EVs were first enriched using ExoMax (SBI, Palo Alto, CA) by combining equal parts culture supernatant and exosome enrichment reagent and incubated overnight. EVs were pelleted and resuspended in PBS. Iodixanol (OptiPrep) gradients were prepared in PBS in 1.2% increments ranging from 6% to 18%. EVs were layered on top of gradient and ultracentrifuged for 90min at 100,000 x g in a SW41 Ti rotor (Beckman, Indianapolis, IN). Gradient fractions were collected from the top of the gradient in 1ml increments. Fractions 9.6% to 14.4% iodixanol were collected and pooled together and diluted with 20ml PBS. Samples were centrifuged at 120,000 x g for 90 min at 4C in Type 70 Ti rotor (Beckman). Resulting EV pellets were resuspended in 200l of PBS.

EV size and concentration were determined using Nanoparticle Tracking Analysis software using a NanoSight NS300 (Malvern, UK). Three video captures of 60 seconds each were used to generate average concentration (EVs/ml) and particle size (nm)(mean± SEM).

For each condition, isolated EVs were adjusted to 1x1012 particles/mL for spin column EVs and to 1x1011 particles/mL for iodixonal isolated EVs. EVs (5 L/tissue block) were applied to fresh *ex vivo* lymphoid tissue blocks at day 0 and day 6. Spin column EVs contained small amounts of HIV-1, thus tissues treated with these EVs were treated with RTV or NVP to prevent unwanted HIV-1 infection. Iodixonal EVs were free of HIV-1 as determined by (i) p24 assessment, and (ii) the absence of HIV-1 replication when applied to lymphoid tissue without addition of ART.

Supernatants from RTV experiments were also subjected to ultracentrifugation (4°C at 26,000 RPM for 70 minutes Ultracentrifuge WX ultra 80, Thermo Fisher) to collect EV-free supernatants. These supernatants were applied to tissues at one part supernatant to two parts fresh medium to determine any effect on cytokine production.

Culture medium alone was divided into EV and EV-free supernatant fractions by ultracentrifugation as described above. EV pellets were resuspended in PBS and added to fresh lymphoid tissue cultures, and supernatants free of EVs were added to fresh lymphoid tissue cultures as described above. EVs were collected from an equivalent volume of culture medium as used for isolation of EVs from conditioned medium.

EVs were also characterized by flow cytometry. To verify the presence of tetraspanins on EVs, we labelled vesicles with Cell Mask Deep Red (CMDR, 0.5g/ml, Thermo Fisher) then captured EVs with magnetic nanoparticles (MNPs) coupled with anti-HLA Class I or CD45 antibodies and stained with CD9-BV421, CD63-AF488, and CD81-PE and analyzed on a BD Symphony instrument as previously described[7], thresholding on CMDR events. Lack of non-EV markers (apolipoprotein A1, apolipoprotein B, and albumin) was verified by labelling EVs with Bodipy FL (0.5M) and staining with biotin labelled antibodies followed by streptavidin-phycoerythrin (SA-PE) (all Thermo Fisher reagents), thresholding on Bodipy FL events.

**Cytokine measurement**

An in-house multiplexed bead-based assay was used to measure the following 33 cytokines: IL (interleukin)-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-16, IL-17, IL-18, IL-21, IL-22, IL-33, Calgranulin A (Calg A or S100A8), Eotaxin (CCL11), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-regulated alpha (GRO-α or CXCL1), interferon- (IFN-), interferon--induced protein (IP-10 or CXCL10), interferon-inducible T-cell alpha chemoattractant (ITAC or CXCL11), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1 or CCL2), monokine induced by IFN- (MIG or CXCL9), macrophage inflammatory protein-1α (MIP-1α or CCL3), MIP-1β (CCL4), MIP-3α (CCL20), regulated on activation normally T-cell expressed and secreted (RANTES or CCL5), transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α).

Antibody pairs and protein standards were purchased from R&D Systems except for IL-4 (Biolegend) and IL-21 (Thermo Fisher). All reagents were verified to be free of cross reactivity. Magnetic Luminex beads with distinct spectral signatures were coupled to cytokine specific capture antibodies according to manufacturer’s recommendations. Standards and samples were diluted in assay buffer (1X PBS with 20 mM Tris-HCl, 1% each normal mouse and goat serum (Gemini Bioproducts) and 0.05% Tween 20) and combined with bead mixtures and incubated overnight at 4°C. Plates were washed and incubated with mixtures of polyclonal biotinylated anti-cytokine antibodies in assay buffer for 1 hour at RT, followed by washing and 30 minute incubation with 16μg/ml SA-PE (Thermo Fisher) in PBS. Plates were washed and beads were resuspended in PBS and read on a Luminex 200 acquiring 100 events per region, and analyzed using Bioplex Manager software (BioRad). Cytokine concentrations were determined using 5P regression algorithms.

**Intracellular cytokine measurement**

Flow cytometry was employed to assess cell types producing the increased IFN-, MIP-1α, MIP-1β, RANTES, and TNF-α. Uninfected or HIV-1-infected tissues were harvested at day 3 of culture, dissociated into single cell suspensions and filtered through 40m strainers, and treated with Brefeldin A (5μg/mL, Biolegend) for 3-4 hours. Cells were stained with A350 for live/dead discrimination, followed by cell surface staining with mouse anti-human antibodies as follows: CD3 BV650, CD4 BV605, CD8 APC-Cy7, CD19 BV786, CD56 BV711, CD123 BV510, CD11c PE-Cy5, HLA-DR PE-CF594, CD14 BB700, CD1c BV421 and CD11b BUV661 (all from BD Biosciences). The cells were then intracellularly stained for IFN- PE-Cy7, MIP-1 PE, MIP-1 APC-A700, RANTES APC, and TNF- BB515 (BD Biosciences). Results are reported as percentages of increase over control tissues. Cell populations were defined as the following: monocytes/macrophages (Lineage−HLA-DR+ CD123−CD14 or CD11b+), mDCs (Lineage−HLA-DR+CD14 lo/- CD123−CD11c+CD1c+), pDCs (Lineage−HLA-DR+ CD14−CD11c−CD123+), NK cells (CD3−CD56+), CD4+ T cells (CD3+CD4+), CD8+ T cells (CD3+CD8+), and B cells (CD3−CD19+).

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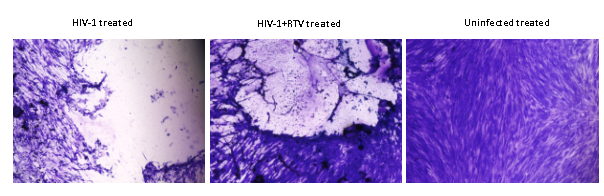
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**SUPPLEMENTAL FIGURES**

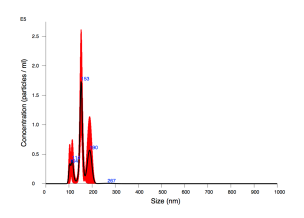
**Supplemental Figure 1.** **Reactivated CMV is cytopathic to MRC5 cells.**

Tissue blocks from uninfected, HIV-1-infected, and HIV-1-infected and RTV treated conditions were collected and frozen at day 12. Tissues blocks were thawed, homogenized and applied to MRC5 cells for four hours at 1:10 dilutions, removed and CMC overlays were added to wells. Cultures were stopped at day 13, overlays were removed, cells were washed, fixed with 10% formalin, stained with crystal violet and cell damage was assessed by microscopy. Two of four tissues showed cytopathic effects, shown is one representative figure.



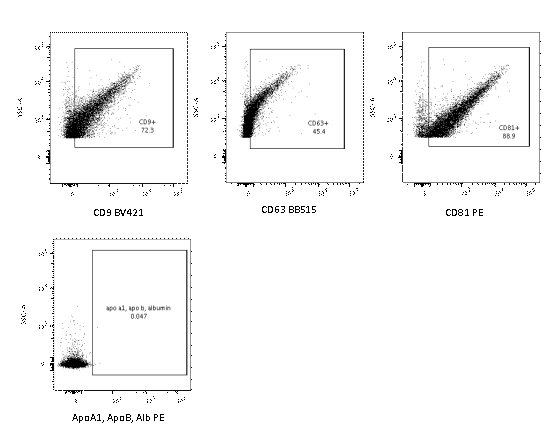
**Supplemental Figure 2.** **NanoSight characterization of isolated EVs.**

EVs isolated by membrane affinity spin columns from pooled tonsil culture supernatants were characterized NanoSight. Three video captures of 60 seconds each were used to generate average concentration and particle size. Shown is representative size distribution for EVs from control tissues at day 9.



**Supplemental Figure 3.** **Flow cytometry characterization of isolated EVs.**

EVs from lymphoid tissue cultures isolated by iodixonal gradients EVs **A)** were labelled with CMDR then captured with magnetic nanoparticles (MNPs) coupled with anti-HLA Class I or CD45 antibodies and stained with CD9-BV421, CD63-AF488, and CD81-PE to identify tetraspanins, and **B)** were labelled with BODIPY FL and interrogated for non-EV markers (apolipoprotein A1, apolipoprotein B, and albumin) by staining with biotin labelled antibodies followed by SA-PE.



**Supplemental Figure 4: Extracellular vesicles from HIV-1 infected human tissues upregulate cytokines.** Extracellular vesicles (EVs) collected at day 9 from donor-matched tissue cultures either infected with HIV-1, infected with HIV-1 and treated with NVP, treated only with NVP or uninfected (controls) were added to previously untreated tissues. EVs were added to cultures at day 0 and day 6 of culture, along with NVP to exclude the possible effect of HIV-1 contamination in EVs collected from HIV-1 infected cultures. (**A**) HIV-1 replication in donor-matched tissues infected with HIV-1 or exposed to EVs. HIV-1 replication was assessed by p24gag released into the supernatant. Presented are mean cumulative releases (± S.E.M.) over a 12 day period.Cytokine release into the culture medium by tissues treated with EVs was measured every three days and cumulative production over 12 days was calculated. Cytokines are presented as pg/ml (± S.E.M.) (\**p*<0.05, n=12) **(B)** IFN-, **(C)** MIP-1, **(D)** MIP-1, **(E)** RANTES, and **(F)** TNF-.

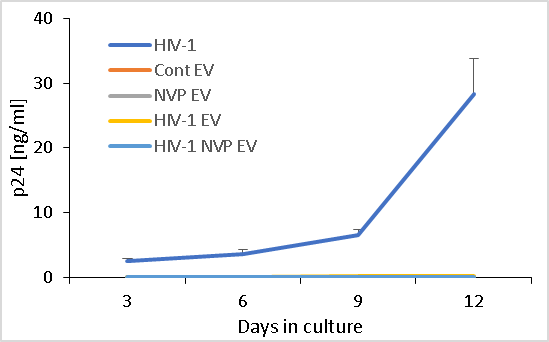
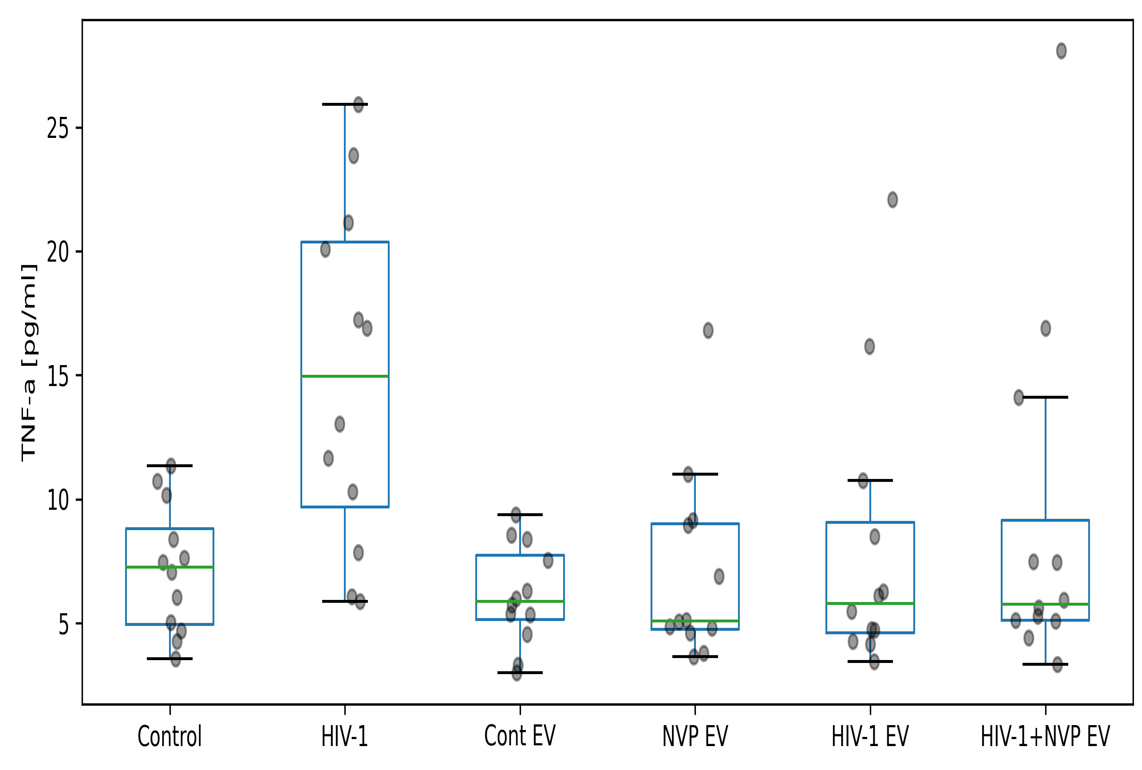
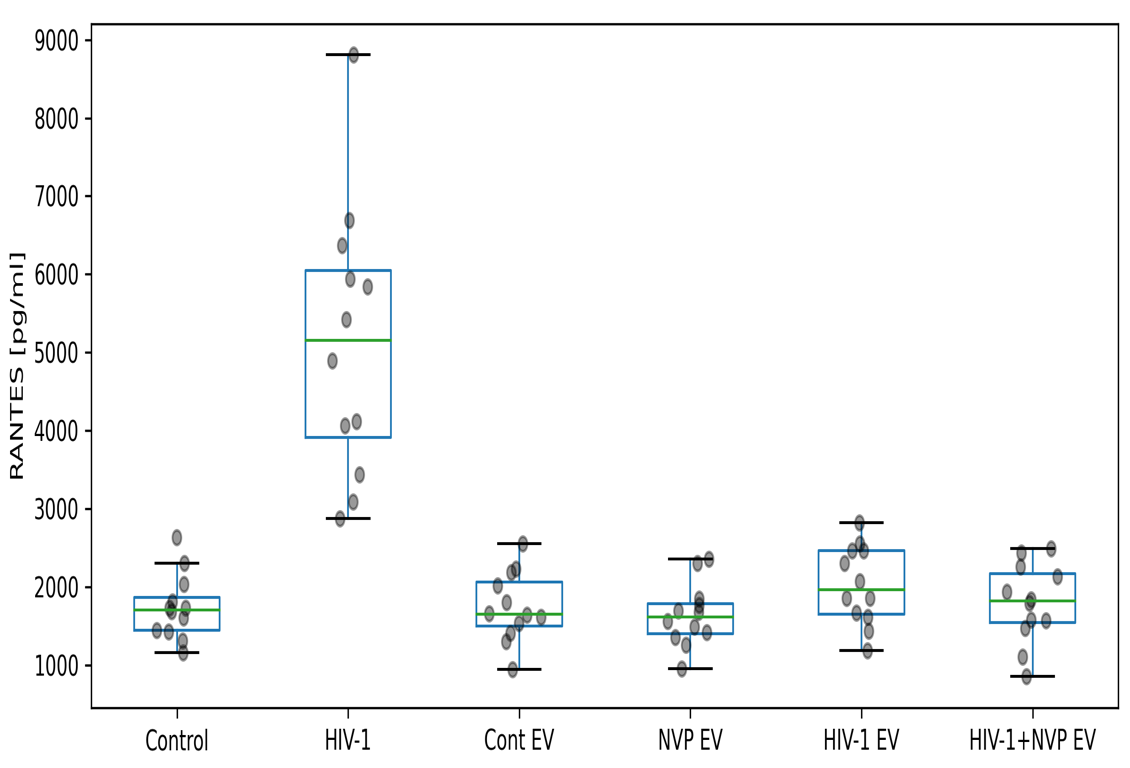
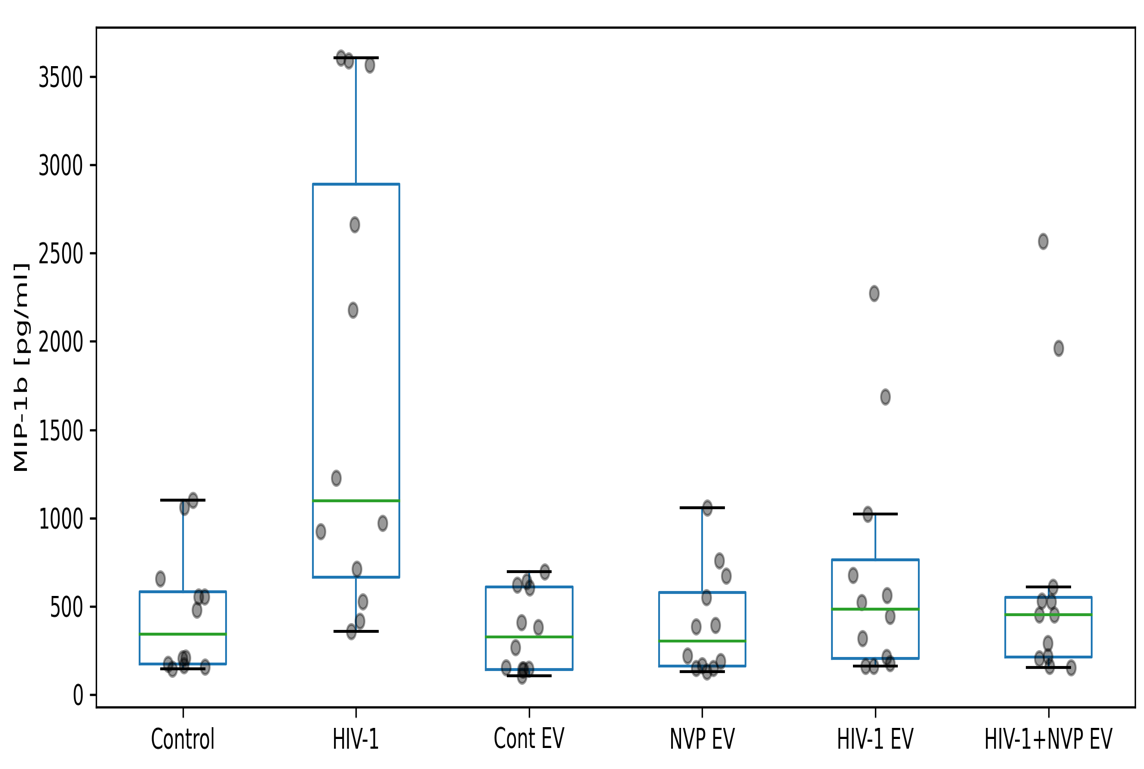
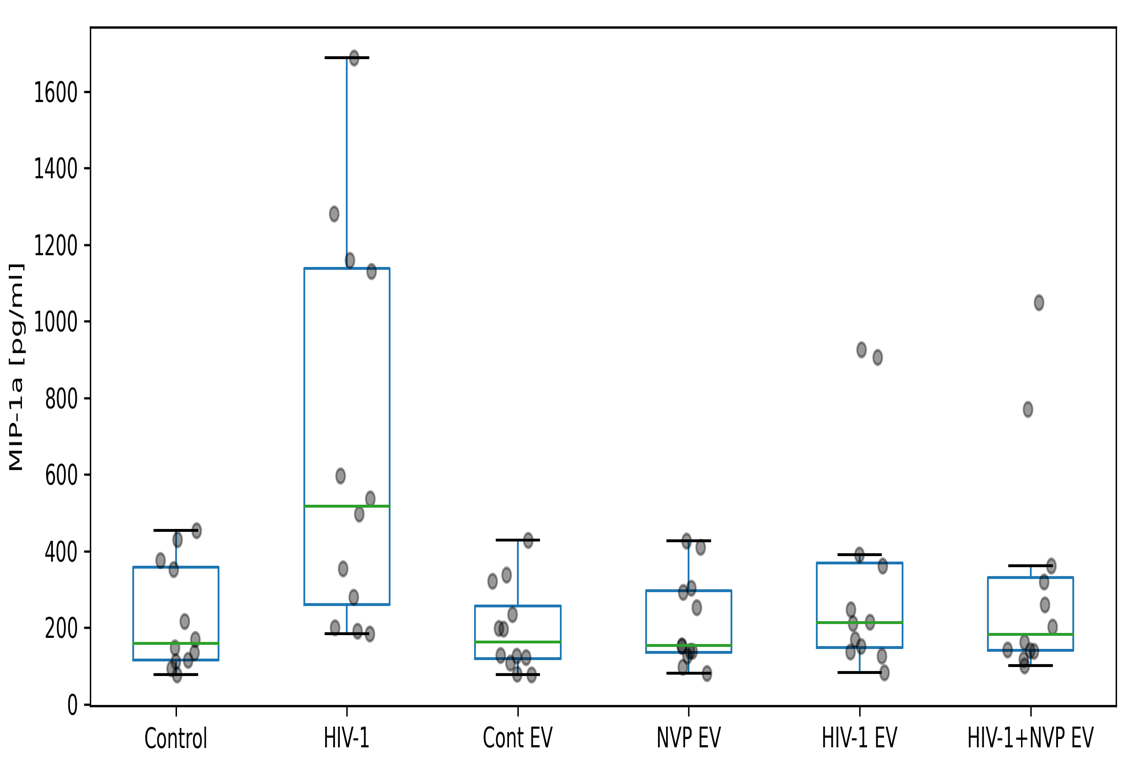
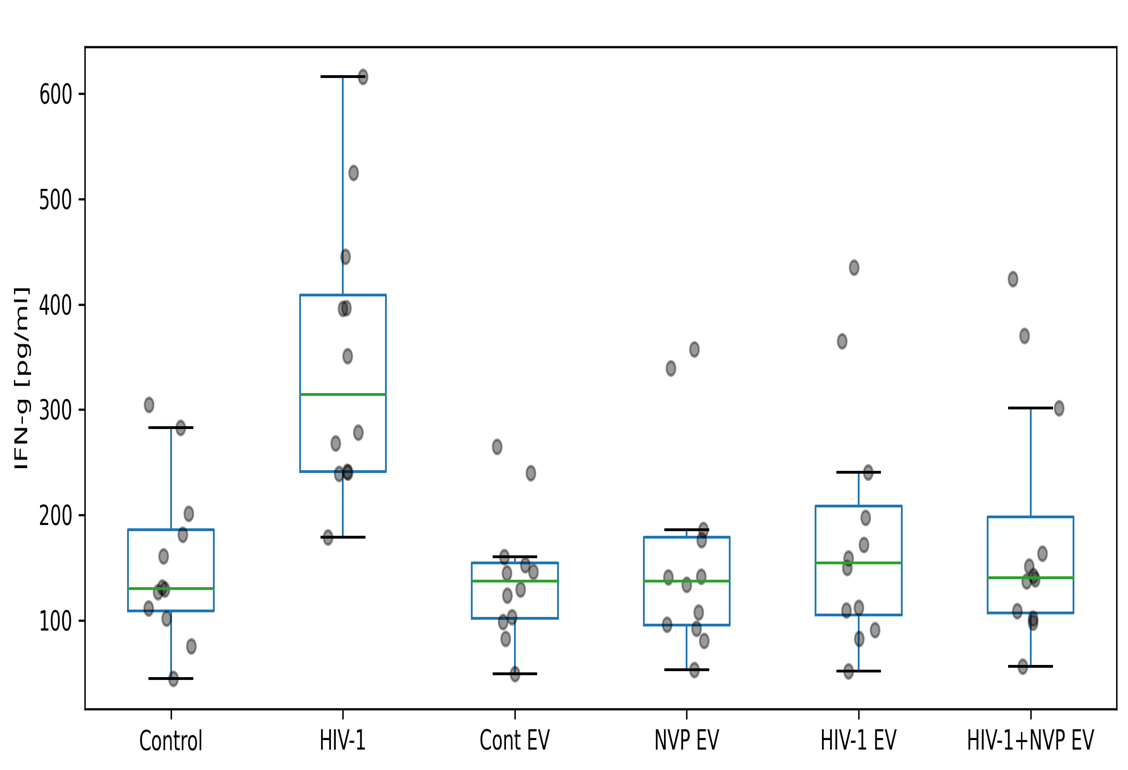
**A**

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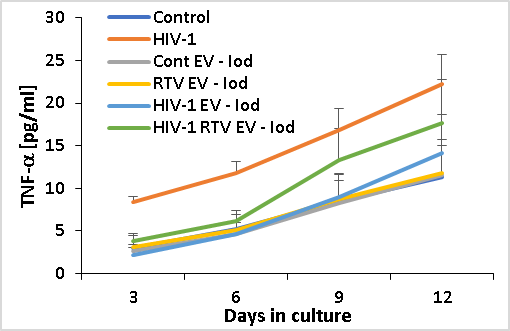
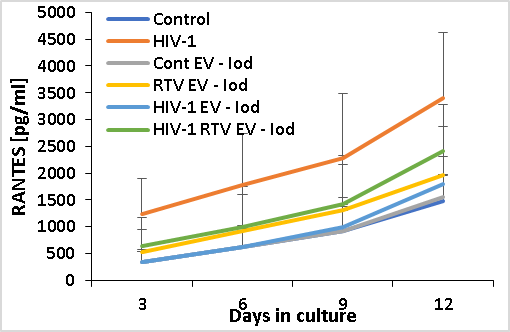
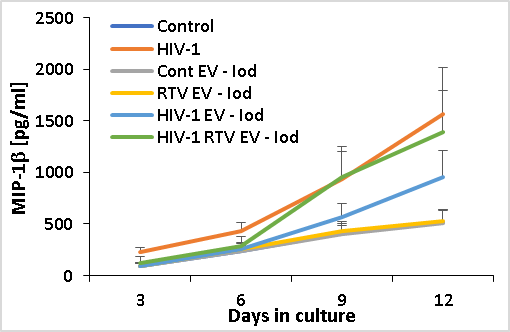
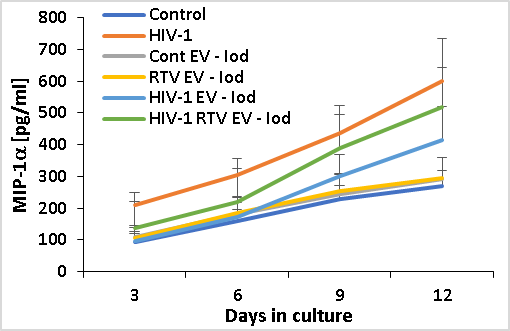
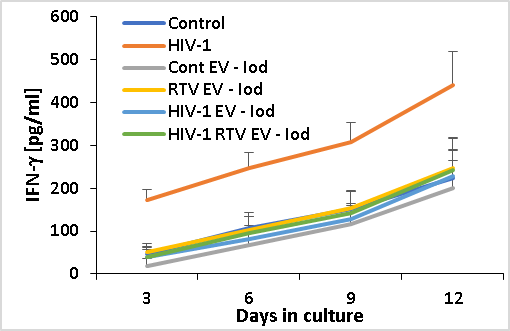
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**Supplemental Figure 5: Human *ex vivo* lymphoid tissues treated with EVs.**

Human lymphoid tissue cultures were untreated (Control), infected with HIV-1, or treated with EVs from day 9 of previously HIV-1 infected tissues (HIV-1 EV-Iod), previously HIV-1 infected tissues treated with RTV (HIV-1 RTV-Iod), uninfected (Cont EV-Iod) or RTV only treated tissues (RTV EV-Iod). EVs were added to cultures at day 0 and 6. Cytokine production was measured every three days and cumulative production is shown for **(A)** IFN-, **(B)** MIP-1, **(C)** MIP-1, **(D)** RANTES, and **(E)** TNF- (Mean ± S.E.M., n=5).



**A**

**B**

**C**

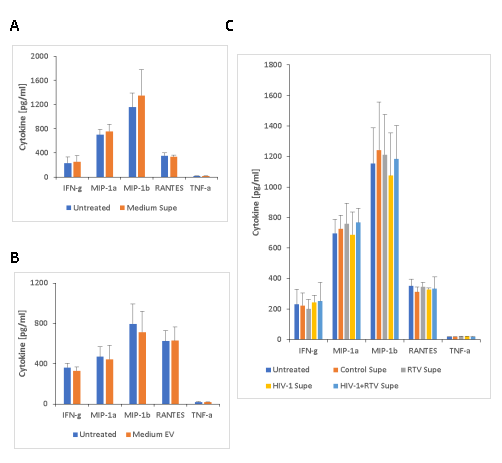
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**Supplemental Figure 6.** **EVs and EV-free supernatant (supes) fractions from culture medium, and supes from experimental conditioned media did not induce cytokine production.**

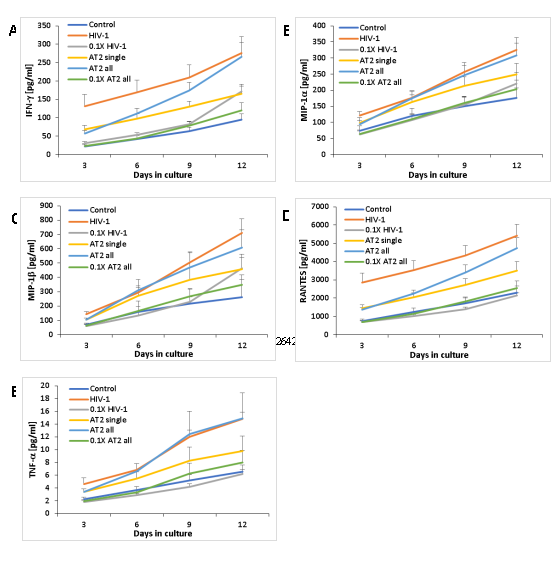
Lymphoid tissue cultures were treated with **A)** EVs and **B)** supe fractions from culture medium separated by ultracentrifugation and compared to untreated tissues, and **C)** supe fractions from control (uninfected), RTV treated, HIV-1-infected, and HIV-1-infected tissues treated with RTV compared to control uninfected and HIV-1-infected. EVs or supes (1:3) were added to cultures at day 0 and 6, along with RTV beginning at day 0, to prevent replication of HIV-1. Cytokine production was measured every three days and cumulative production is shown for IFN-, MIP-1, MIP-1, RANTES, andTNF- (n=3.



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**Supplemental Figure 7: Cytokine production by tissues treated with AT-2 inactivated HIV-1**

Tissues untreated (Control) or treated with a single inoculum at day 0 of HIV-1, a single equivalent inoculum of AT-2 HIV (HIV-1 single exp), equivalent inoculums of AT-2 X4LAI04 every three days (AT-2 HIV-1), 0.1X AT-2 virus every three days (AT-2 HIV-1 (0.1X), or medium treated with AT-2 as a control (AT-2 medium). Cytokine production was measured every three days and cumulative production is shown for **(A)** IFN-, **(B)** MIP-1, **(C)** MIP-1, **(D)** RANTES, and **(E)** TNF- (Mean ± S.E.M., n=9.

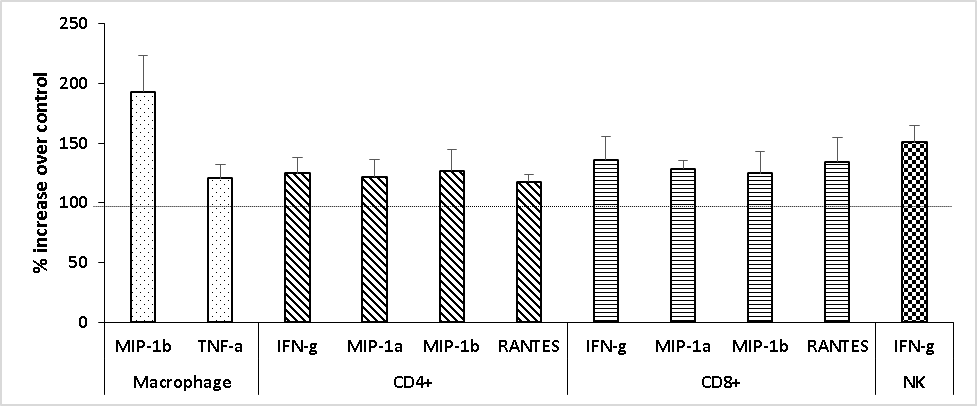


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**Supplemental Figure 8.** **Upregulated cytokines are produced by a variety of cells types.**

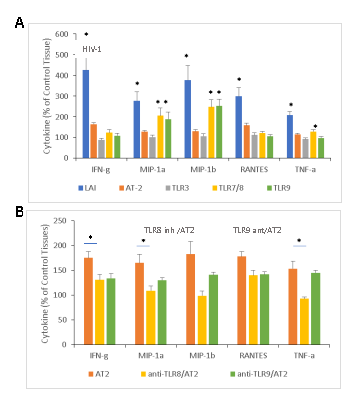
Flow cytometry was employed to assess cell types producing the increased IFN-, MIP-1α, MIP-1β, RANTES, and TNF-α. Uninfected or HIV-1-infected tissues were harvested at day 3 of culture, dissociated into single cell suspensions, treated with Brefeldin A and stained for live/dead discrimination. Cell surface staining was used to phenotype cells, and intracellular staining identified cells producing IFN-, MIP-1, MIP-1, RANTES, and TNF-. Results are reported as percent of increase over control tissues for different cell subsets (n=4).

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**Supplemental Figure 9.** TLR ligands play partial role in cytokine upregulation.

Tissues were treated **A)** at day 0 with HIV-1, AT2 inactivated HIV-1, or 1 g/ml of TLR agonists: TLR3 poly(I:C), TLR7/8 (R848), or TLR9 (ODN2006 CpG-B)(\*p<0.05, n=9). Alternatively, tissues were pretreated **B)** with TLR8 inhibitor (CU-CPT9a) or TLR9 antagonist (ODN TTAGGG) for 4 hours followed by challenge with AT-2 HIV-1 or AT-2 inactivated HIV-1 only (AT2). Cumulative cytokine production over 12 days, expressed as a percent of matched control tissues, is shown (\*p<0.05, n=7).



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