Online Data Supplement.

HIV gp120 in Lungs of ART-Treated Individuals Impairs Alveolar Macrophage Responses To Pneumococci

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Figure E1 A

Figure E1 B

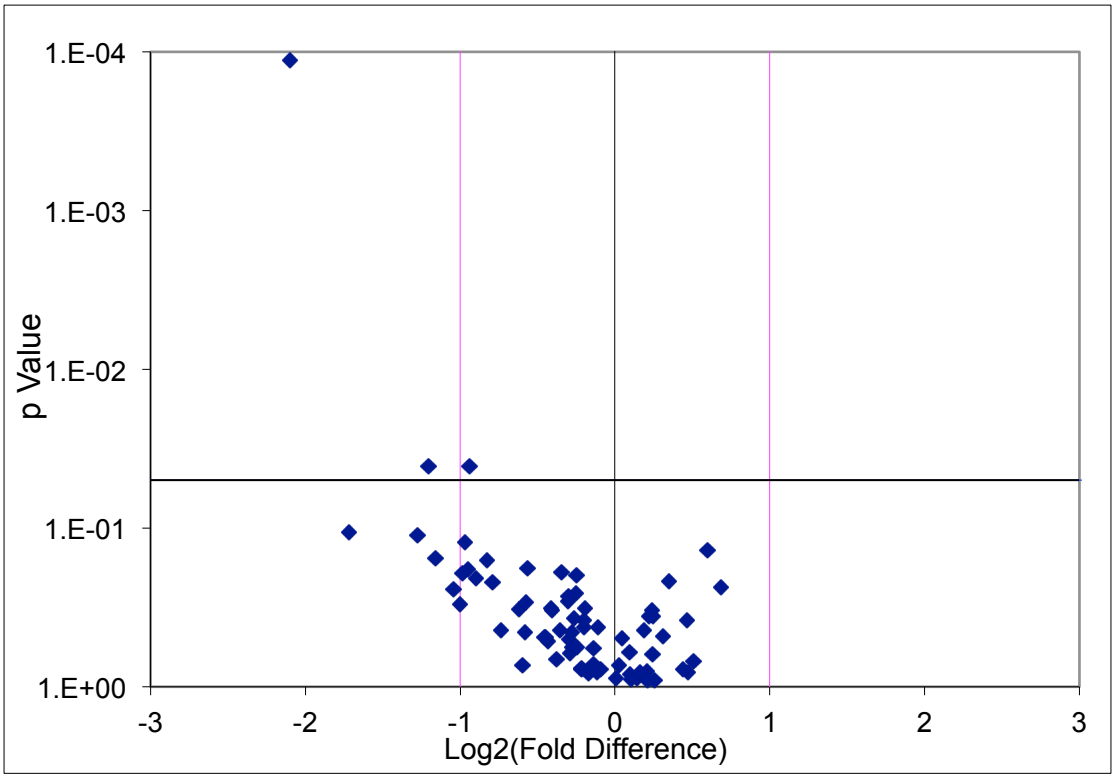
1.E-04
1.E-03
1.E-02
1.E-01
1.E+00
p Value
-3
-2
-1
0
1
2
3
Log2(Fold Difference)

Figure E1 C

GOI	FC	p value	q value	GOI	FC	p value	q value
HUWE1	-0.28	0.5659	0.8380	AKT3	-0.17	0.8210	0.8925
EEF2K	-0.38	0.6728	0.8925	PTEN	-0.34	0.1908	0.8111
TNFSF10	0.21	0.7973	0.8925	GSK3B	-0.14	0.5662	0.8380
FASLG	-0.29	0.6158	0.8711	IL15	0.60	0.1382	0.8111
TNFRSF10A	-0.43	0.5189	0.8170	IL1B	-1.05	0.2440	0.8111
TNFRSF10B	-0.30	0.5056	0.8134	CCL22	-0.74	0.4409	0.8111
TNFRSF10C	0.47	0.8173	0.8925	CCL3	-0.83	0.1612	0.8111
TNFRSF10D	-0.22	0.7651	0.8925	CCL4	-0.95	0.1813	0.8111
SPATA2	-0.62	0.3269	0.8111	CXCL10	0.44	0.7700	0.8925
TNF	0.47	0.3832	0.8111	IL10	-0.97	0.1225	0.8111
CD209	-0.94	0.0410	0.8111	CCL15	0.51	0.6940	0.8925
IL12B	-0.25	0.1971	0.8111	CCL2	-2.10	0.0001	0.0084
NFE2L2	-0.19	0.3231	0.8111	CXCL3	-1.00	0.3025	0.8111
MAP3K5	-0.25	0.5640	0.8380	IL1A	-1.28	0.1117	0.8111
CFLAR	-0.14	0.7259	0.8925	IL6	-1.16	0.1548	0.8111
CX3CL1	-0.40	0.3283	0.8111	IL8	-0.36	0.4420	0.8111
BCL2L1	-0.30	0.2891	0.8111	MMP12	-0.98	0.1914	0.8111
BCL2	-0.56	0.1792	0.8111	PTGS2	0.09	0.6070	0.8711
BAX	-0.20	0.4219	0.8111	RIPK2	0.24	0.6239	0.8711
BAD	-0.25	0.2586	0.8111	CCL5	-0.58	0.2935	0.8111
MCL1	0.24	0.3300	0.8111	CD80	0.10	0.8322	0.8925
BID	0.35	0.2162	0.8111	CD86	0.14	0.8856	0.9138
BCL2A1	0.19	0.4446	0.8111	CXCL11	0.25	0.9014	0.9138
TP53	-0.45	0.4850	0.8134	CXCL9	-0.79	0.2180	0.8111
BBC3	-0.14	0.7603	0.8925	GBP5	0.21	0.9161	0.9161
PMAIP1	0.03	0.7314	0.8925	TNFSF10	0.16	0.8150	0.8925
APAF1	-0.58	0.4490	0.8111	CCR5	-0.60	0.7328	0.8925
PERP	-0.11	0.4243	0.8111	CD36	0.69	0.2358	0.8111
IER3	-1.72	0.1054	0.8111	MMP2	-1.21	0.0405	0.8111
USP9X	-0.20	0.3800	0.8111	MMP7	-0.90	0.2073	0.8111
EEF2	-0.30	0.2687	0.8111	SOD1	0.24	0.3573	0.8111
FAS	0.01	0.8849	0.9138	SOD2	-0.42	0.3195	0.8111
BCL2L11	-0.46	0.4904	0.8134	GSR	-0.09	0.7709	0.8925
FADD	0.10	0.8947	0.9138	TXN	0.31	0.4794	0.8134
PAK2	-0.28	0.4494	0.8111	HMOX1	0.04	0.4948	0.8134
AKT1	-0.27	0.3726	0.8111	NQO1	0.22	0.3599	0.8111
AKT2	-0.22	0.7812	0.8925	GPX2	-0.12	0.8083	0.8925

Supplemental Figure Legends

Figure E1 The expression of genes associated with macrophage activation and apoptosis regulation is not altered in alveolar macrophages in people living with HIV.

Expression levels of genes regulating macrophage activation and apoptosis in overnight rested alveolar macrophages (AM) from control (n=5) and ART treated HIV-1$^+$ (n=8) donors, measured by customized RT^{2}Profiler PCR array (Qiagen) and normalized to 11 housekeeping genes (A). The mean Log2 fold difference in CT value (ΔΔCT) between HIV-1 and control donors is plotted against Log p value (B). Entrez Gene Official Symbols for housekeeping genes and genes of interest (GOI) are listed with the fold change in expression (FC) in AM from ART treated HIV-1$^+$. p values are calculated based on a Student's t-test of the replicate 2^(- ΔCt) values for each gene in the control group and treatment groups and q values calculated with a false discovery rate of 1% (C).

Figure E2 Flow cytometry gating strategy illustrating no alteration in alveolar macrophage surface markers associated with polarization but altered pulmonary CD4$^+$:CD8$^+$ T cell ratio in HIV.

Bronchoalveolar lavage (BAL) cells from control (n=6) and ART treated HIV-1$^+$ (ART, n=11) donors were enriched for alveolar macrophages (AM) by overnight plastic adhesion. AM were identified as CD206$^+$ and surface expression of CD80 (M1), CD206, CD163 and CD200r (M2) compared between groups by flow cytometry (A). Representative histograms for each surface marker conjugate

(shaded) and isotype control (empty) are shown for a healthy donor (control) and ART treated HIV-1$^+$ donor (B). AM were identified on forward scatter (FSC) and side scatter (SCC), laser voltages were set such that APC/PE isotype controls were in the first log**,** CD206 expression measured on the FL4 channel and CD80, CD163 or CD200r expression measured on FL2 and the MFI of each antibody conjugate and its isotype control were compared to derive the geometric mean ratio (GMR) (C). 24 h after BAL, non-adherent cells were washed and labeled with anti-CD3-PE, anti -CD4-APC, anti-CD8-brilliant violet, and a viability dye. Upper panel: Lymphocytes were identified by forward (FSC) and side scatter (SSC) and doublets excluded using FSC-area (FSC-A) /FSC-height (FSC-H). Events expressing >1 $\log_{10}$ higher than unstained on UV 450/40 (UV live dead) were considered dead and excluded. Viable cells with high blue 575/26 (PE) expression were gated as CD3$^+$. CD3$^+$ cells expressing high red 660/20 (APC) and low violet 450/50 (brilliant violet) were classified as CD4$^+$ T cells and those with low red 660/20 and high violet 450/50 were classified as CD8$^+$ T cells. Cells incubated with PE, APC and brilliant violet conjugated isotype control antibodies are shown for comparison in the lower panel. Events numbers on each gate are percentages of parent gate. Representative plots from an ART donor are shown with those from a control donor on the right hand end (D). Box plots show median, IQR and range. MFI= geometric mean fluorescence intensity, IC=isotype control, APC = allophycocyanin, PE = phycoerythrin.

Supplemental Figure E3 Co-Culture with $CD8^+$ T-cells does not modulate macrophage apoptosis or intracellular bacterial killing

Monocyte-derived macrophages (MDM) were co-cultured on their own or with autologous $CD8^+$ lymphocytes that were either treated with control beads or activated with Dynabeads Human T-Activator CD3/CD28 for 2 h. MDM were then mock-infected (MI) or challenged with *Streptococcus pneumoniae* (D39) and the number of viable MDM (A), the percentage of fragmented or condensed nuclei (B) or intracellular bacterial survival at 4 h and 20 h estimated (C) n=4. ns = no significant interaction for CD8 co-culture, 2 way ANOVA.

Supplemental Materials and Methods

Volunteers

Alveolar macrophages (AM) and lymphocytes were isolated from bronchoalveolar lavage (BAL) fluid obtained from healthy, never smoker, hepatitis B and C virus negative HIV-1 seropositive and HIV-seronegative volunteers (Table I). HIV-1-seropositive individuals were recruited from the HIV clinics of Sheffield Teaching Hospitals. 14 were established on ART and 13 had used either Non Nucleoside Reverse Transcriptase Inhibitors (NNRTI) (7 donors) or Protease Inhibitors (PI) (6 donors) exclusively as the third agent in their ART regimen. 3 ART naïve patients were also included for evaluation of BAL cell counts, HIV RNA and gp120 measurements only. Whole blood for human peripheral blood mononuclear cell (PBMC) isolation was collected from healthy volunteer donors.

Bacteria Type 2 *S. pneumoniae* (D39 strain, NCTC7466) were grown to mid log phase as previously described (2). D39 were opsonized in RPMI (Sigma-Aldrich) containing 10% anti-pneumococcal immune serum, with detectable levels of antibody, as previously described (2). Opsonized D39 at a multiplicity of infection (MOI) of 10 (unless otherwise stated in the Figure legends) or media alone (mock infection) were added to wells and incubated at 4^{0}C for 1 h (to maximize adherence) and then at 37^{0}C for 3 h (to maximize internalization). Cultures were washed 3 times in PBS to remove non-adherent bacteria, then incubated for a further 12-16 h in RPMI 1640 (Lonza) with 10% decomplemented fetal bovine serum (FBS; Bioclear). In certain experiments, either 10-100 ng/mL recombinant HIV-$1_{LAI/IIIB}$ envelope glycoprotein gp120 (obtained through the Programme EVA Centre for AIDS Reagents, NIBSC, HPA, UK from ImmunoDiagnostics

Inc. MA, USA) or autologous lymphocytes were added to MDM from 1-2 h prior to infection and after washing at 4 h.

Virus. CCR5 tropic HIV-1_{BaL} (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1_{BaL} from Dr. Suzanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo) was propagated for 3-4 d in IL-2 (PeproTech) maintained peripheral blood lymphocytes (PBL) and then 7 d in MDM differentiated in RPMI + 10% autologous human serum with 20 ng/mL macrophage colony-stimulating factor (M-CSF, R&D Systems). Culture supernatants were ultracentrifuged through a 20% sucrose buffer and re-suspended in 10% FBS-RPMI after each passage (3). Replication competent HIV-1 virus preparations were titrated on the NP2 astrocytoma cell line stably transfected with CD4 and CCR5 and stained for intracellular p24 (3).

Isolation and culture of macrophages and other leukocytes. PBMC were isolated by Ficoll-Paque (Pharmacia-Amersham) density centrifugation of whole blood from healthy donors as described previously (2). PBMC were plated at 2 x 10^6 cells/mL in RPMI 1640 media with 2 mmol/L L-glutamine (Gibco BRL) containing 10% human AB serum (First Link) in 24-well plates (Costar). After 24 h, non-adherent cells were removed and adherent cells were cultured in in 5% CO_2 at 37^oC in either 10% FBS-RPMI for 14 days. Prior to use, representative wells were scraped to determine the concentration of MDM prior to challenge with D39 or HIV-1_{BaL} Peripheral blood lymphocytes (PBL) were purified from PBMC by performing 2 plastic adherence steps to remove monocytes. For HIV-1_{BaL} propagation PBL were re-suspended at 1 x 10^6 cells/mL in RPMI 1640 +10% AB serum with 20 µg/mL interleukin (IL)-2 (PeproTech) and 0.5 mg/mL phytohemagglutinin (PHA, Sigma-Aldrich). For syngeneic co-culture experiments, PBL were enriched for $CD8^+$ T cells by negative selection using the Easy Sep Human $CD8^+$ T

cell enrichment Kit (Stem cell technologies) as per the manufacturer's instructions and re-suspended at 1 x 10^6 cells/mL. Purity of >95% was confirmed by flowcytometry. $CD8^+$ T cells were activated over 2 h using Dynabeads Human T-Activator CD3/CD28 (Life technologies) according to the manufacturer's instructions then added 1:1 to MDM cultures from the same donor for D39 challenge.

AM were isolated as previously described (4). Briefly, lavage with ≤200 mL of warm sterile saline was carried out after the bronchoscope was lodged in a middle-lobe subsegmental bronchus +/- midazolam sedation by a consultant respiratory physician, and BAL fluid was aspirated under 23kPa suction pressure and collected in a pre-cooled trap (Argyle™, Coviden). BAL fluid volume was documented then sieved through sterile gauze, centrifuged at 400g x 10 minutes and supernatant frozen at -80 ^oC. The pellets were resuspended in RPMI 1640 + 10% AB serum + 40 u/mL penicillin (Lonza) + 40 μgmL^{-1} streptomycin (Lonza) + 0.5 µg/mL amphotericin (Fungizone™, GIBCO) at a density of 2 x 10^5 AM/mL. 100 µL was diluted 1:1 with HIFCS and fixed to prepare a cytospin slide. Suspensions that contained visible red blood cells were subjected to Ficoll-Paque density centrifugation and re-suspended. Cells were incubated overnight at 37°C in 5% CO_2 in 6, 24 and 96 well cell culture plates (Costar). The following day medium with non-adherent cells was replaced with fresh antibiotic free FBS-RPMI. The non-adherent cells from each donor were pooled and prepared for flow analyses. Adherent AM were cultured until use on the third day of incubation.

HIV infection of MDM. 7 day MDM were inoculated with doses of HIV-1_{BaL} equivalent to MOI of 0.1-1.0 or sham virus (prepared from the same, uninoculated, PBL/MDM propagation) for 16 h and then incubated in fresh FBS-RPMI for a further 7 d.

Intracellular p24 staining. NP2, MDM or AM were fixed and permeabilized in an ice-cold mixture 1:1 of pure acetone and methanol, washed and incubated with 1:25 p24 antibody (IgG1κ monoclonal antibody to HIV-1 gag p24, code no. E366, obtained through the Programme EVA Centre for AIDS Reagents, NIBSC, UK from Dr B Wahren) then 5 μg/mL goat anti-mouse antibody conjugated to β-galactosidase (Southern Biotechnology Associates), each for 1 h, then overnight at 37 ^{o}C in a galactosidase substrate solution of 0.5 mgmL^{-1} 5-bromo-4-chloro-3-indolyl—galactopyranoside (X-gal, Melford) in PBS containing 3 mmol/L potassium ferricyanide (FLUKA), 3 mmol/L potassium ferrocyanide (FLUKA) and 1 mmol/L magnesium chloride (Sigma). Blue stained cells positive for p24 were counted by microscope to provide a virus titre or number and proportion of infected cells (3).

SDS-PAGE and Western blotting. Whole cell extracts were lysed on ice in buffer containing 20mM TRIS-HCl pH7.4, 5mM ethylenediaminetetraacetic acid (EDTA), mM ethylene glycol tetraacetic acid (EGTA), 150mM NaCl and 1% sodium dodecyl sulphate (SDS), with protease inhibitor cocktail (Complete™, Roche). Protein was quantified using a modified Lowry protocol (DC Protein Assay, Biorad) and protein was loaded equally per lane and separated by 12% SDS-PAGE then blotted onto nitrocellulose membranes (Bio-Rad Laboratories) with protein transfer confirmed by Ponceau S staining. Blots were blocked for 60 min at room temperature in PBS containing 0.05% Tween with 5% (v/w) skim milk powder then incubated overnight with anti-Mcl-1 (rabbit polyclonal, 1:1000 S-19, Santa Cruz, recognizing full length Mcl-1, 40 kDa and ubiquitinated Mcl-1,

>40 kDa) or anti-ubiquitin (Pierce Scientific 1:500) or anti-USP9X (rabbit polyclonal, 1:2500, Bethyl Laboratories) with anti-tubulin (mouse monoclonal, 1:2000, Sigma-Aldrich) or anti-actin (rabbit polyclonal, 1:5000 Sigma-Aldrich) as loading controls. Proteins were detected using HRP-conjugated secondary antibodies (1:2000; Dako) and enhanced chemiluminescence (ECL) (Amersham Pharmacia). The density of bands was measured using ImageJ™ software v1.440 (NIH). Fold change from mock-infected was calculated and normalized to the fold change in loading control (2, 5).

Ubiquitin pull-down assay. Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) and ubiquitinated proteins were isolated immediately using an enrichment kit for ubiquitin (Thermo Scientific) according to the manufacturer's instructions. Levels of ubiquitin were analyzed by Western blot.

Flowcytometry. Unless otherwise stated, all analyses were performed by FACSCalibur flow cytometer (BD Biosciences) and at least 10,000 cells were analyzed for each condition. To detect loss of $\Delta\psi m$ at 16 h cells were stained in 250µL RPMI containing 10µM 5,5′,6,6-tetrachloro-1,1,3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1, Molecular probes) for 15 min, washed, scraped and analyzed. Loss of $\Delta\psi m$ was demonstrated by a loss of fluorescence on the FL-2 channel as previously described (2). AM and lymphocyte surface marker expression was measured by incubating cells with 100 mg/ml human IgG1 (Sigma) to block Fcγ receptors then for at least 30 minutes at 4^0C with fluorophore conjugated antibodies and appropriate isotype controls at concentrations of 0.1-0.25 µg per 10^5 cells in 100µL of 0.1% BSA in PBS (FACS buffer) according to the manufacturers' instructions as follows. AM were dually stained with

mouse anti-human CD206, (19.2), (APC), (eBioscience), and either mouse anti-human CD163, (GHI/61), (PE), (eBioscience), mouse anti-human CD80, (2D10.4), (PE), (eBioscience) or mouse anti-human CD200r, (OX108), (PE), (eBioscience). Gates were set on FSC/SSC to exclude debris and identify intact cells. APC ($CD206^+$) geometric mean fluorescence intensity (MFI) expression was measured on this subpopulation using the FL4-H channel. PE ($CD80^+$ / $CD163^+$ / $CD200r^+$) MFI for each conjugated antibody was measured on the $CD206^+$ gated subpopulation using the FL2-H channel (Supplemental Figure 2B). Values were expressed as the ratio of the MFI of the marker (APC or PE) to the MFI of the isotype control. Lymphocytes were stained with mouse anti-human anti-CD3 (SK7) phycoerythrin (PE), (eBioscience), mouse anti-Human CD38 (HB7), fluorescein isothiocyanate (FITC), (eBioscience), mouse anti-human anti-CD4 (S3.5), allophycocyanin (APC), (Invitrogen) and mouse anti-human CD8, (RPA-T8), (Brilliant Violet 421), (Biolegend) for lymphocytes and LIVE/DEAD® Blue Fixable Dead Cell Stain Kit (L23105, Molecular Probes, Invitrogen). In parallel anti-mouse Ig kappa and negative control compensation beads (BD™ Compbeads, BD Biosciences) were incubated with each antibody conjugate separately. Labeled cells were then analyzed on a 13 color LSRII™ (BD Biosciences) flow cytometer. The beads were used to set a compensation matrix, unstained cells were used to set FSC and SSC, and isotype control labeled cells used to set the red 633nm (660/20 filter, APC), blue 488 nm (575/26 filter, PE and 530/30 filter FITC), violet 405nm (450/40 filter, brilliant violet), and UV 355nm (450/40 filter UV) laser voltages and filters. These were then kept the same for each subsequent donor sample. Lymphocytes were identified on FSC/SSC. Doublet cells were excluded using a FSC-A versus FSC-H event plot. Cells with high UV 450/40 (LIVE/DEAD® Blue) intensity on the singlet cell gate were considered to be dead lymphocytes and excluded. T lymphocytes were identified as $CD3^+$ cells, defined as blue

575/26^{+} events in the live cell population. Back gating was performed to confirm that the CD3^{+} cells were within the original lymphocyte gate on FSC/SSC. CD4^{-}/CD8^{+} (CD8^{+} T lymphocyte) cells were defined as violet 450/40^{+} red 660/20^{-} events and CD4^{+}/CD8^{-} (CD4^{+} T lymphocyte) cells were defined as violet 450/40^{-} red 660/20^{+} within the CD3^{+} population (Supplemental Figure 2D). The expression of CD38 on CD8^{+} T cells was defined as the ratio of the MFI on the blue 530/30 channel for CD3^{+}/CD4^{-} /CD8^{+} gated events to that of isotype control. Data analyses were performed using FlowJo™ software version 9.3.2 (Tree Star, Inc.).

Measurement of Mitochondrial Reactive Oxygen Species (mROS). mROS production was measured at 16 h by incubating macrophages with 2.5 μM MitoSOX™ Red (Invitrogen) for 15 min at 37°C. This cell permeable dye contains dihydroethidine which targets mitochondria and undergoes O_2^{-} -dependent hydroxylation to 2-hydroxyethidium which fluoresces at excitation/emission spectra of 400/590nm. Cells were washed with Hank's Balanced Salt Solution (HBSS, Gibco), and fluorescence measured on scraped cells by FACSCalibur using 488 nm excitation to measure oxidized MitoSOX™ Red in the FL2 channel. Because MitoSOX™ Red cannot be used on fixed cells HIV-1/sham infected MDM were analyzed directly using a Varioskan Flash multimode reader (Thermo Scientific) in containment level 3 conditions. To control for number of mitochondria, cellular mitochondrial mass was measured in matched wells using MitoTracker™ Green FM (Invitrogen).

Microscopy. *Apoptosis detection.* Nuclear morphology was examined by in 4', 6-diamidino-2-phenylindole (DAPI, Vectorshield™, Vector Laboratories) using a fluorescent light microscope (Leica, DMRB 1000) at 1000x magnification using a 100x/1.30 (PL Fluotar) objective at room temperature. Blinded reviewers counted 300

cells on duplicate coverslips mounted on glass slides for the presence of condensed or fragmented nuclei, to estimate apoptosis as previously described (2). MDM treated with 5μM staurosporine (Sigma-Aldrich) were used as a positive control. *BAL cell identification*. BAL cells were identified using light microscopy (Nikon, Eclipse TE300) of Diff-Quick stained (Dade Behring) cytospins at 1000x magnification using a 100x/1.25 oil emersion (Nikon Plan) objective. At least 300 cells were counted. *HIV-1$_{BaL}$ MDM imaging*. MDM stained for p24 were imaged with a Leica DMRB microscope at 400x magnification using a 40x objective and imaging software (SPOT Advanced Imaging Software).

Caspase activation. Macrophage caspase 3/7 activity was measured directly in culture wells at 16 h using the Caspase-Glo™ 3/7 assay (Promega) in accordance with the manufacturer's instructions. Luminescence was measured on a Varioskan Flash multimode reader (Thermo Scientific).

Quantification of gp120 by ELISA. gp120 in BAL was quantified by ELISA. High Binding plates (Costar) were coated with 1mg/mL with each of three human monoclonal Abs against gp120: 14E, 17B, EH21 (kindly provided by James E Robinson, Tulane University, New Orleans). After blocking with 1% ovalbumin, BAL fluid supernatants were concentrated by approximately 12 fold using 50k Amicon Ultra filter (Merck Millipore) and added to the plate and gp120 detected using a 1/2000 dilution of the same biotinylated antibodies (6). Two-fold serial dilutions of recombinant gp120 (HIV-1$_{LAI/IIIB}$) were used as standards and results considered positive if above the limit of detection in the linear range of a log/lin standard curve, giving a lower limit of detection in the ELISA of 25ng/mL and BAL fluid of 2ng/mL. BAL fluid samples from 6

HIV-1-seronegative donors were used as negative controls or spiked with recombinant gp120 for positive controls.

Intracellular Bacterial Killing assay. Assessment of intracellular bacterial viability was carried out at 4 h and 20 h as previously described (7). Briefly, cells were infected and at 4 h washed x 3 in PBS then incubated for 30 minutes in fresh medium containing 40 units/mL benzyl penicillin (Crystapen™, Genus Pharmaceuticals) and 20 μg/mL gentamicin (Cidomycin™, Sanofi) to kill extracellular bacteria before being lysed with 2% saponin (Sigma) for 12 min. Lysates were diluted to 1ml in PBS, and intracellular bacterial numbers determined by Miles-Misra surface viable count. Alternatively following penicillin/gentamicin treatment cells were returned to the incubator in medium containing 0.7 μg/mL vancomycin (Sigma) to ensure extracellular bacteria remained undetectable with an antimicrobial that lacked significant intracellular penetration then washed and lysed at 20 h and viable counts performed as before.

Real-time measurement of cell respiration. Macrophage mediated real-time mitochondrial respiration (e.g. OXPHOS) was measured by the XF24 extracellular flux analyzer (Seahorse, Bioscience). Briefly, 14 day MDM were detached from T27 culture flasks using accutase (Biolegend) and gentle scraping and re-seeded at 2x105 well in an XF24 cell plate (Seahorse Bioscience) and left to re-adhere. Following pneumococcal challenge in the presence or absence of gp120 wells were washed with XF medium (Seahorse, Bioscience) that had been supplemented with 4.5g/L D-glucose, 2mM L-glutamine, 1.0mM Na-pyruvate and penicillin (100U/mL and streptomycin (100μg/mL) and adjusted to pH 7.4 with 1.0M NaOH and then incubated for an hour at 37°C without CO2 in 630μL/well of the same XF medium with or without gp120. The XF24 utility plate was submerged in XF calibrant (Bioscience) and incubated for 16 h at 37°C. The ATP

synthase inhibitor oligomycin A (70µL at 15µM), the mitochondrial uncoupler FCCP (77µL at 20µM) and the combination of the complex I inhibitor rotenone and complex III inhibitor antimycin A (85µL at 10µM) were added to the cartridge containing injection ports A, B and C respectively and incubated for an hour at 37°C without CO_2 supplementation. An XF24 analyser was then used to measure the rate of oxygen consumption (OCR) and extracellular acidification (ECAR) kinetically before and after injecting oligomycin (1.5µM, the final concentration), FCCP (2.0 µM) and rotenone (1.0 µM) plus antimycin A (1.0µM) (Sigma Aldrich) as per the manufacturer's instructions. Cells were then lysed with mammalian cell lysis buffer (Thermofisher) plus protease inhibitors cocktail (Roche) and the total protein was estimated by the Bradford method and kits (Bio-Rad). Baseline ECAR, basal OCR, ATP linked OCR, maximum respiration capacity and mitochondrial inner membrane mediated proton leak were calculated from ECAR and OCR measurement after normalization for protein content using the formula described by Zhang J et al. (8).

RT-PCR Array. After 48 h in culture AM were washed 3 times to remove non-adherent cells, a technique which has been demonstrated to yield a purity of 98% viable AM (4), then harvested in Tri Reagent (Sigma) and preserved at -80°C. Total RNA was extracted using a Direct Zol RNA miniprep kit (Zymo research). Nucleic acid concentration was measured using a Nanodrop spectrophotometer (ThermoFisher Scientific) and RNA quality (RNA integrity number) was assessed with an Agilent BioAnalyzer following the Nano Kit Lab-On-A-Chip procedure (Agilent Technologies). The cDNA template was synthesized from RNA with a RIN > 7.5 with a RT^2 First Strand Kit (SABiosciences) and cDNA product visualized by Agarose Gel Electrophoresis following PCR amplification with a housekeeping gene (β actin) primer. cDNA was analyzed for expression of genes

associated with apoptosis and macrophage activation and 11 housekeeping genes (Supplemental Figure I) with a custom made primer/probe sets on a RT^2 Profiler PCR Array (SABiosciences) using the Mx3000P QPCR System (Agilent). Ct values were gathered and data analysis was performed via the ΔΔCt method using PCRArrayDataanalysis_V4 software (SABiosciences) to determine relative expression differences between the comparison groups with reference to the housekeeping genes. Changes of mRNA abundances by 2-fold and higher with a p value <0.05 calculated based on a Student's t-test of the replicate 2^(- Delta Ct) values for each gene in the control group and treatment groups, and a q value calculated by correcting for multiple testing using the method of Benjamini and Hochberg (1) with a false discovery rate of 1%, were considered significantly different between the comparison groups.

Ultra-sensitive detection of HIV-1 RNA in BAL. BAL HIV-1 RNA was quantified using a modified version of the Abbott Real Time HIV-1 assay (Maidenhead, UK), following ultracentrifugation of up to 12 ml of BAL at 240,000 g for 20min at 4^{o}C, and resuspension of the pellet in 1 ml of the supernatant, similarly to what has been recently applied in plasma samples (9). Modified assay sensitivity was determined by spiking 12 ml of acellular BAL obtained from HIV-negative volunteers with the World Health Organization (WHO) 3rd International HIV-1 RNA Standard (NIBSC code:10/152, Hertfordshire, UK) at concentrations of 1 and 8 copies/ml in triplicate. The sensitive protocol showed a lower limit of detection (LLD) 1 copy/ml, which ranged from 1-2 copies per mL (cps/mL) depending on the initial input volume of BAL. Inhibition in BAL samples was also tested using acellular BAL supernatants and plasma obtained from HIV-seronegative volunteers spiked in parallel with four dilutions (100, 500, 1000, 5000

and 10000 copies/ml) of the WHO 3rd International HIV-1 RNA Standard and tested in duplicate. No inhibition occurred when testing BAL samples. *Sample measurement:* Up to 12mL (median 12ml; IQR: 10.25, 12.00) of each BAL sample was ultracentrifuged and HIV-1 RNA was quantified using the sensitive protocol. 7 of 13 (54%) samples had sufficient volume to allow testing in duplicate.

Statistics

Results are recorded as mean and SEM unless otherwise stated. Sample sizes were informed by standard errors obtained from similar assays in prior publications (2, 10). Decisions on use of parametric or non-parametric tests were based upon results of D'Agostino-Pearson normality tests. Parametric or nonparametric testing was performed with the indicated tests using Prism 6.0 software (GraphPad Inc.). Comparisons between two conditions were performed using a paired or unpaired t-test for parametric data, or a Mann-Whitney U test or Wilcoxon signed rank test for non-parametric data. Correlation was measured with two-tailed Pearson. When two or more conditions were assessed in two experimental groups (e.g. HIV-1-seropositive vs. HIV-seronegative), data were analyzed by ANOVA with Holm-Sidak post-tests. Significance was defined as $p < 0.05$.

Supplemental References

1. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *J Roy Stat Soc B Met* 1995; 57: 289-300.

2. Marriott HM, Bingle CD, Read RC, Braley KE, Kroemer G, Hellewell PG, Craig RW, Whyte MK, Dockrell DH. Dynamic changes in Mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance. *J Clin Invest* 2005; 115: 359-368.

3. Tsang J, Chain BM, Miller RF, Webb BL, Barclay W, Towers GJ, Katz DR, Noursadeghi M. HIV-1 infection of macrophages is dependent on evasion of innate immune cellular activation. *AIDS* 2009; 23: 2255-2263.

4. Gordon SB, Molyneux ME, Boeree MJ, Kanyanda S, Chaponda M, Squire SB, Read RC. Opsonic phagocytosis of Streptococcus pneumoniae by alveolar macrophages is not impaired in human immunodeficiency virus-infected Malawian adults. *J Infect Dis* 2001; 184: 1345-1349.

5. Bewley MA, Marriott HM, Tulone C, Francis SE, Mitchell TJ, Read RC, Chain B, Kroemer G, Whyte MK, Dockrell DH. A cardinal role for cathepsin d in co-ordinating the host-mediated apoptosis of macrophages and killing of pneumococci. *PLoS Pathog* 2011; 7: e1001262.

6. Rychert J, Strick D, Bazner S, Robinson J, Rosenberg E. Detection of HIV gp120 in plasma during early HIV infection is associated with increased proinflammatory and immunoregulatory cytokines. *AIDS Res Hum Retroviruses* 2010; 26: 1139-1145.

7. Marriott HM, Ali F, Read RC, Mitchell TJ, Whyte MK, Dockrell DH. Nitric oxide levels regulate macrophage commitment to apoptosis or necrosis during pneumococcal infection. *FASEB J* 2004; 18: 1126-1128.

8. Zhang J, Nuebel E, Wisidagama DR, Setoguchi K, Hong JS, Van Horn CM, Imam SS, Vergnes L, Malone CS, Koehler CM, Teitell MA. Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. *Nat Protoc* 2012; 7: 1068-1085.

9. Ruggiero A, De Spiegelaere W, Cozzi-Lepri A, Kiselinova M, Pollakis G, Beloukas A, Vandekerckhove L, Strain M, Richman D, Phillips A, Geretti AM, Group ES. During Stably Suppressive Antiretroviral Therapy Integrated HIV-1 DNA Load in Peripheral Blood is Associated with the Frequency of CD8 Cells Expressing HLA-DR/DP/DQ. *EBioMedicine* 2015; 2: 1153-1159.

10. Bewley MA, Preston JA, Mohasin M, Marriott HM, Budd RC, Swales J, Collini P, Greaves DR, Craig RW, Brightling CE, Donnelly LE, Barnes PJ, Singh D, Shapiro SD, Whyte MKB, Dockrell DH. Impaired Mitochondrial Microbicidal Responses in Chronic Obstructive Pulmonary Disease Macrophages. *Am J Respir Crit Care Med* 2017.