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Title Page

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

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Author contributions

PC and DD conceived this work. Experiments were designed/performed by PC, MB, MM, RR and HM. NM and RM provided technical assistance with HIV-1 infection of macrophages. AM, AB and AP designed and performed ultrasensitive HIV-1 RNA measurement. All authors contributed to preparation and review of the manuscript.

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At a glance summary:

Scientific Knowledge on the Subject:

Why people living with HIV who are on treatment remain at much greater risk of pneumococcal disease remains unclear.

What This Study Adds to the Field:

This study finds that, despite antiretroviral therapy there is persistent low-level viral replication in the lung. Alveolar macrophages from people living with HIV-1 demonstrate a defect in pneumococcal killing, which is caused by the HIV-1 glycoprotein gp120. This results in reduced susceptibility to macrophage apoptosis, a necessary component for bacterial killing.

This article has an online data supplement, which contains supplemental figures (E1-3) and a detailed description of all materials and methods and is accessible from this issue's table of content online at <u>www.atsjournals.org</u>

Structured Abstract

Rationale

People living with HIV (PLWH) are at significantly increased risk of invasive pneumococcal disease, despite long-term antiretroviral therapy (ART). The mechanism explaining this observation remains undefined.

Objectives

We hypothesized apoptosis-associated microbicidal mechanisms, required to clear intracellular pneumococci that survive initial phagolysosomal killing, are perturbed.

Methods

Alveolar macrophages (AM) were obtained by bronchoalveolar lavage (BAL) from healthy donors or HIV-1-seropositive donors on long-term ART with undetectable plasma viral load. Monocyte-derived macrophages (MDM) were obtained from healthy donors and infected with HIV-1_{BaL} or treated with gp120. Macrophages were challenged with opsonized serotype 2 *Streptococcus pneumoniae* and assessed for apoptosis, bactericidal activity, protein expression and mitochondrial reactive oxygen species (mROS). AM phenotyping, ultra-sensitive HIV-1 RNA quantification and gp120 measurement were also performed in BAL.

Measurements and Main Results

HIV-1_{BaL} infection impaired apoptosis, induction of mROS and pneumococcal killing by MDM. Apoptosis-associated pneumococcal killing was also reduced in AM from ART treated HIV-1-seropositive donors. BAL fluid from these individuals demonstrated persistent lung $CD8^{+}$ T-cell lymphocytosis, and gp120 or HIV-1 RNA was also detected. Despite this, transcriptional activity in AM freshly isolated from PLWH was broadly similar to healthy volunteers. Instead, gp120 phenocopied the defect in pneumococcal killing in healthy MDM through post-translational modification of Mcl-1, preventing apoptosis induction, caspase activation and increased mROS generation. Moreover gp120 also inhibited mROS dependent pneumococcal killing in MDM.

Conclusions.

Despite ART, HIV-1, via gp120, drives persisting innate immune defects in AM microbicidal mechanisms, enhancing susceptibility to pneumococcal disease.

Abstract Word Count 241

<u>Introduction</u>

HIV-1-seropositive individuals have a significantly increased risk of pneumococcal disease that persists despite antiretroviral therapy (ART), even after $CD4^+$ T-cell reconstitution (1, 2). Alveolar macrophages (AM) are essential for pneumococcal clearance from the lung (3) yet evidence of modulation of AM immune competence against pneumococci by HIV-1 has proven elusive; opsonic phagocytosis of pneumococci is preserved during HIV-1 infection (4) and while defective phagolysosomal killing is reported for some pathogens it has not been demonstrated for pneumococci (5).

The capacity of healthy human tissue macrophages to destroy extracellular bacteria through internalization and phagolysosomal killing is finite (6) and AM need to engage a second, delayed microbicidal strategy involving apoptosis-associated killing to eliminate residual viable intracellular pneumococci, which involves combinations of reactive oxygen species (ROS) and nitric oxide (NO) (3, 7-9). The apoptotic program is regulated by the anti-apoptotic Bcl-2 protein Mcl-

1 and induction of a mitochondrial apoptosis pathway (10). Apoptosis-associated killing enhances clearance of pneumococci, limits tissue invasion and downregulates the inflammatory response in the lung (10, 11). Importantly, HIV-1 is associated with an anti-apoptotic gene expression profile in monocytes *in vivo* and promotes macrophage resistance to apoptosis, which contributes to these cells constituting a viral reservoir for HIV-1 (12-15).

We addressed whether HIV-1 prevents engagement of the apoptotic program required for pneumococcal killing. Here we report a selective deficit in delayed, apoptosis-associated pneumococcal killing in AM from ART-treated HIV-1-seropositive volunteers. We document evidence of low level viral replication and gp120 detection in the lung despite long-term suppressive ART and confirm that HIV-1 envelope glycoprotein gp120 is sufficient to inhibit macrophage killing of pneumococci in human monocyte-derived macrophage (MDM), through altered post-translational regulation of Mcl-1 and failure to induce mitochondrial ROS (mROS) generation.

Some of the results of these studies have been previously reported in the form of an abstract and doctoral thesis (16, 17).

Materials and Methods

Additional detail on the method for making these measurements is provided in an online data supplement.

Bacteria, Virus and Infections

Opsonized, type 2 *S. pneumoniae* (D39 strain, NCTC7466) were used for infection of macrophages at a multiplicity of infection of 10 unless otherwise stated, as described (10). In some infections autologous peripheral blood lymphocytes (PBL), or HIV-$1_{LAI/IIIB}$ envelope glycoprotein gp120 (NIBSC, UK) at 10-100 ng/mL were added to MDM. HIV-1_{BaL} (NIH AIDS Reagent Program,) was propagated in PBL, then MDM and purified before cell inoculation. Infection rates were measured by intracellular p24 staining as described (18).

Volunteers

Healthy, never smoker, hepatitis B and C virus negative, HIV-1-seropositive patients either established on ART or ART naïve (used as comparator for BAL and virology studies), were recruited from the HIV clinic of STH for bronchoscopy along with matched HIV-seronegative volunteers, described in Table 1.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy donors and differentiated to MDM(10). Non-adherent PBMC were enriched for $CD8^+$ T-lymphocytes by negative selection and >95% purity confirmed by flowcytometry. $CD8^+$ T-lymphocytes were added 1:1 to MDM. Cells were isolated from bronchoalveolar lavage (BAL) fluid as described (4).

Western blot

Whole cell extracts were isolated using SDS-lysis buffer and separated by SDS gel electrophoresis.

Flow Cytometry

Cell surface marker expression was measured by flow cytometry with fluorophore conjugated antibodies or isotype controls. MDM mROS was measured using MitoSOX-Red (Invitrogen), and loss of $\Delta\psi_m$ with JC-1 (Molecular probes).

Microscopy

Nuclear fragmentation and condensation indicative of apoptosis were detected using 4′6′-diamidino-2-phenylindole (DAPI)(10). BAL cells were identified on stained cytospins.

Caspase activation

Cellular caspase activity was measured using Caspase-Glo 3/7 (Promega) according to the manufacturer's instructions. Luminescence was measured on a Varioskan Flash microplate analyzer (Thermo Scientific).

Quantification of gp120

BAL supernatants were concentrated using 50k Amicon Ultra-filters (Merck Millipore) and gp120 quantified with human monoclonal anti-gp120 antibodies (14E, 17B and EH21), using recombinant gp120 (HIV-$1_{LAI/IIIB}$) for standards, by ELISA, as described (19).

Metabolic measurements

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the XF24 extracellular flux analyser (Seahorse, Bioscience) as described (20).

RT-PCR Array

AM gene expression was measured after 48 h with a custom made RT^2 Profiler PCR Array (SABiosciences) using QPCR.

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 similarly to methods in plasma samples (21). After confirming no inhibition, sensitivity was determined at 1-2 copies per mL by spiking acellular HIV-negative BAL with World Health Organization 3rd International HIV-1 RNA Standard (NIBSC, UK).

Statistics

Results are recorded as mean and SEM unless stated. Sample sizes were informed by standard errors obtained from similar assays in prior publications (10, 20). Analysis was performed with tests, as outlined in the figure legends, using Prism 6.0 software (GraphPad Inc.) and significance defined as $p<0.05$. Decisions on use of parametric or non-parametric tests were informed by the distribution of the data.

Results

HIV-1 inhibits delayed pneumococcal killing by macrophages

To examine whether HIV-1 influences macrophage killing of pneumococci we infected MDM with HIV-1_{BaL}, an M-tropic stain of HIV-1 (18) or sham virus (Figure 1A) and then, after adjusting for cell numbers, challenged MDM with pneumococci. The numbers of viable intracellular bacteria in MDM 4 h post bacterial challenge, which are the net result of opsonic phagocytosis and phagolysosomal killing (4), were unaltered by HIV-1_{BaL} (Figure 1B). By contrast, 20 h after pneumococcal challenge the intracellular bacterial load was higher in HIV-1_{BaL} MDM (Figure 1C). When we examined engagement of the MDM apoptotic programme we found caspase 3/7 activation, development of apoptotic nuclei and loss of cell numbers following pneumococcal challenge were significantly reduced by HIV-1_{BaL} compared to sham infection (Figure 1D-F). Mcl-1 was down-regulated in sham virus exposed MDM but levels were preserved in HIV-1_{BaL} MDM (Figure 1G-H). Despite comparable mitochondrial density HIV-1_{BaL} MDM had elevated production of mROS after mock-infection but, unlike sham virus exposed MDM, failed to upregulate mROS after pneumococcal challenge (Figure 1I-J). Overall these findings support a specific deficit in the delayed apoptosis-associated phase of pneumococcal killing in HIV-1_{BaL} MDM.

Impaired apoptosis-associated pneumococcal killing in alveolar macrophages from HIV-1-seropositive individuals treated with ART.

We next investigated whether alveolar macrophages (AM) from the unique lung environment of asymptomatic HIV-1-seropositive individuals established on ART with undetectable plasma HIV-1 viral RNA (Table 1), would also demonstrate impaired pneumococcal clearance. In line with HIV-1_{BaL} infected MDM, AM from ART treated HIV-1-seropositive donors showed a selective defect in delayed pneumococcal killing at 20 h (Figure 2A-B). AM in these samples also showed reductions in caspase 3/7 activation, numbers of apoptotic nuclei and cell loss relative to healthy controls (Figure 2C -E). The impairment of apoptosis following pneumococcal challenge was not related to use of protease inhibitors or non-nucleoside reverse transcriptase inhibitors as the third ART agent (Figure 2F). When we investigated the relationship between the number of HIV-1_{BaL} infected MDM and apoptosis induction following pneumococcal challenge we found no correlation (Figure 2G).

Activation status of AM from HIV-1-seropositive individuals treated with ART is similar to healthy volunteers.

We next investigated if steady state expression of representative genes associated with apoptosis and polarization was altered in AM from our donor groups. Using quantitative PCR arrays we found that while there was an overall trend towards downregualtion of gene expression in AM from ART treated HIV-1-seropositive individuals compared with healthy controls, no consistent differences in the expression of these genes was observed (supplemental Figure E1). Furthermore, representative markers of macrophage polarization states CD80 (M1), CD163, CD206 and CD200r (M2) also showed no significant alteration in surface expression in AM from HIV-1-seropositive individuals on ART (supplemental Figure E2).

Impaired bacterial clearance by alveolar macrophages is associated with markers of viral persistence in the lungs of HIV-1-seropositive individuals on ART.

As pulmonary T-lymphocytes influence macrophage-mediated responses to pneumococci in the airway (22), we next sought evidence of alterations to T-lymphocyte numbers in the airway of the asymptomatic HIV-1-seropositive individuals on ART that might link HIV indirectly to the observed AM phenotype. We first analyzed the BAL cell content and included 3 ART-naive HIV-1-seropositive individuals. Both ART-naïve individuals and those receiving ART had increased lymphocyte numbers in BAL fluid (Figure 3A). Compared to healthy controls, ART-treated HIV-1-seropositive individuals also had a lower percentage of $CD4^+$ T-lymphocytes yet a higher proportion of $CD8^+$ T-lymphocytes and lower $CD4^+$:$CD8^+$ T-lymphocyte ratio (Figure 3B-D, Table 1 and supplemental Figure E2). In addition, we found that the ratio of $CD4^+$:$CD8^+$ T-lymphocytes in BAL correlated with the induction of AM apoptosis, following pneumococcal challenge (Figure 3F). We next explored whether T-lymphocyte CD38 expression, a marker of immune activation in HIV-1 that correlates with viral load (23), was increased in the ART-treated HIV-1-seropositive donors. However, $CD8^+$ T-lymphocytes showed no difference in CD38 expression (Figure 3E). We also tested whether *in vitro* activated, autologous $CD8^+$ T-cells, could alter MDM engagement of apoptosis-associated killing but found no modulation of MDM viability, apoptosis or intracellular bacterial survival (supplemental Figure E3A-C).

The CD4:CD8 ratio in ART-treated HIV-1-seropositive individuals is inversely related to the size of the HIV-1 reservoir in the peripheral blood (24). Therefore we considered an alternative possibility that BAL CD4:CD8 ratio was a marker of persistent HIV-1 replication in the lung; we detected HIV-1 p24 in AM cultures from 2/2 ART-naïve and 3/10 ART-treated HIV-1-seropositive donors respectively (Figure 3G). Using ultrasensitive assays HIV-1 RNA was detected at 79 copies/mL and 1-4 copies/mL of cell free BAL fluid supernatants from 1/1 ART-naïve and 2/13 (15.4%) ART-treated HIV-1-seropositive donors respectively. However, the number of donors with detectable p24 or RNA were too few to determine any correlation between these markers of HIV replication and the BAL CD4:CD8 ratio.

gp120 impairs bacterial killing by reducing macrophage susceptibility to apoptosis following pneumococcal challenge

We detected HIV-1 envelope glycoprotein (gp120) in a 10–100ng/mL range in BAL fluid from five of 11 (45.5%) of the ART-treated and in one of two ART-naïve HIV-1-seropositive donors tested, and observed that those on ART with detectable gp120 also had significantly lower peripheral blood $CD4^+$ counts (Figure 4A). Recombinant gp120 recapitulated the selective deficit in delayed phase pneumococcal killing by MDM (Figure 4B-C) and reduced both numbers of apoptotic nuclei and caspase 3/7 activation following pneumococcal challenge (Figure 4D-E). gp120 was also associated with a baseline increase in mROS, without altering mitochondrial density, but gp120 exposed MDM failed to upregulate mROS after pneumococcal challenge (Figure 4F-G). mROS production was abrogated by MitoTEMPO, a mitochondria-targeted superoxide dismutase mimetic that possesses superoxide and alkyl radical scavenging properties, confirming mitochondria as the source of ROS (Figure 4F).

When we analyzed the bioenergetic response of MDM we observed that pneumococcal challenge led to an increase in baseline extracellular acidification rate (ECAR) and a reduction in maximal oxygen consumption rate (OCR Max), and this switch in metabolism was unaltered by gp120 (Figure 4I-L).

Pneumococcal challenge resulted in increased proton leak across the inner mitochondrial membrane (Figure 4M). However, this response and the loss of mitochondrial inner transmembrane potential ($\Delta\psi m$) were diminished by gp120 (Figure 4H).

We next analysed whether abrogation of mROS upregulation, with an mROS inhibitor MitoTEMPO, altered intracellular pneumococcal killing. After challenging MDM with pneumococci we observed no difference in the number of viable intracellular bacteria after 4 h in the presence of gp120 or mitoTEMPO. However, addition of MitoTEMPO to control MDM increased bacterial survival at

20 h to the same level seen with gp120, but had no effect on viability in gp120 exposed MDM at two distinct multiplicities of infection (Figure 5).

gp120 impairs macrophage apoptosis by altering the post-translational modification of Mcl-1

gp120 prevented downregulation of Mcl-1 (Figure 6A-B) and reduced ubiquitination of Mcl-1 after pneumococcal challenge (Figure 6C-D). Ubiquitination of Mcl-1 is tightly regulated and ubiquitination is reversed by the de-ubiquitinase (DUB) USP9X (25). We detected decreased expression of USP9X following pneumococcal challenge in control MDM but treatment with gp120 abrogated this response (Figure 6E-F).

Discussion

Here we demonstrate for the first time that HIV-1 impairs pneumococcal killing by macrophages. We show HIV-1 is associated with specific defects in the late phase of pneumococcal killing by impairing apoptosis induction and reducing caspase-dependent induction of mROS. Critically, we find this defect in AM from HIV-1-seropositive individuals established on long-term antiretroviral therapy with good immune reconstitution. Furthermore, despite extended periods of ART we find evidence of altered cellular immune responses, viral replication and release of the HIV-1 envelope glycoprotein gp120 in the lungs. gp120 is sufficient to reprise the deficit in pneumococcal killing and does so via altered post-translational regulation of Mcl-1, a key regulator of macrophage apoptosis.

AM are essential for pneumococcal clearance; they initially resist pro-apoptotic stimuli while engaging phagolysosomal bacterial killing but subsequently activate apoptosis, which facilitates bacterial clearance whilst minimizing inflammation (3, 10). We found that HIV-1_{BaL} impaired host-mediated MDM apoptosis during pneumococcal infection and this was associated with a failure to clear internalized pneumococci. Mcl-1 levels were maintained in the HIV-1_{BaL} infected MDM following pneumococcal challenge while caspase 3/7 activation was reduced, indicating that the mitochondrial pathway of apoptosis, implicated in bacterial killing, was impaired (3, 10). This extends prior observations implicating HIV-1 in altered regulation of Bcl-2 family proteins (13, 26).

Caspase 3 activation promotes release of mROS by inhibiting the mitochondrial electron transport complex I and has been identified as a requirement for the increment of mROS generation that is required to mediate apoptosis-associated killing of intracellular pneumococci (20, 27). The failure of HIV-1_{BaL} infected MDM to increase mROS production over baseline following pneumococcal challenge resulted in pneumococcal survival, similar to recent observations in AM from COPD patients (20). In contrast to the requirement for a late increment in mROS to achieve optimal intracellular killing, chronic baseline elevation of mROS, following HIV-1 or gp120 exposure, does not seem to enhance intracellular bacterial killing. Consistent with this an inhibitor of mROS had no impact on early intracellular bacterial viability at 4 h. COPD AM also show chronic baseline elevation of intracellular mROS but no enhancement of early intracellular bacterial killing (20). To play a role in intracellular killing mROS needs to be generated in proximity to bacteria in phagolysosomes (20, 28) and be produced at levels above baseline following caspase 3 activation to overwhelm anti-oxidant systems (20). In COPD there is not only reduced caspase 3/7 activation but also an altered balance between mROS generation and superoxide dismutase (SOD) 2 expression, which suggests increased ability to neutralize baseline mROS. Recent observations show gp120 also upregulates SOD in microglia (29). It is noteworthy that, like COPD, HIV-1 has been associated with chronic increases in oxidative stress in mononuclear phagocytes, despite antiretroviral therapy (30, 31), and adaptions to this in both conditions are predicted to impair the capacity to generate a microbicidal response.

HIV-1 infects and replicates in macrophages and, while establishing a long-lived cellular viral reservoir (15), induces resistance to apoptosis (12, 26). Our finding that HIV-1 infection is linked to intrinsic impairments in macrophage apoptotic responses is supported by previous studies with *Mycobacterium tuberculosis* (32), but to the best of our knowledge ours is the first report of impaired killing of pneumococci or any other acute extracellular bacterial infection. Crucially, we have confirmed our findings in clinically relevant AM from aviraemic HIV-1-seropositive individuals.

Untreated, HIV leads to AIDS and increased rates of opportunistic infection, including bacterial pneumonia and IPD (33). Although ART inhibits viral replication, reconstitutes cell mediated immunity and dramatically reduces opportunistic infection, IPD remains 35 fold and bacterial pneumonia 20 fold more common in HIV-1-seropositive individuals in the era of ART (2, 34-36). Our findings suggest that persisting defects in the macrophage microbicidal response contribute to this risk of pneumococcal disease.

We hypothesized that the observed reductions in delayed bacterial killing were due to indirect effects of HIV-1; only a minority of AM in ART-naïve individuals are infected with HIV-1 (37) and, furthermore, within 24 weeks of ART initiation there are large reductions in both BAL fluid RNA and cell-associated HIV-1 nucleic acid (38). Our volunteers had received a median of 75 months ART and had no HIV-1 RNA detectable in peripheral blood by standard assays. Our *in vitro* MDM model allows manipulation of the percentage of MDM that are positive in a culture (18) and we saw no association between the rate of direct MDM HIV-1 infection and apoptosis. Macrophage effector functions are influenced by their activation status (39) and AM from ART-naïve HIV-1-seropositive individuals show classical (M1) activation (37, 40, 41). However, when we measured the activation status and transcriptome of AM from our virally suppressed HIV-1 donors we found no difference from healthy controls. While the plasticity of macrophages makes it conceivable that differences in activation and gene transcription could be lost during AM isolation and culture (40, 42), we conclude that once established on long-term ART, HIV-1 seropositive have no persisting changes in transcriptional pathways regulating AM activation.

T-lymphocytes influence early macrophage-mediated innate immune responses to pneumococci in the airway (22). Consistent with prior reports (38) ART-naïve individuals had increased lymphocyte numbers in BAL fluid but, surprisingly, we also observed persistent lymphocytosis in the BAL of individuals receiving ART. Furthermore, they had a lower CD4:CD8 T-lymphocyte ratio that correlated with altered AM apoptosis. This is a noteworthy finding since low CD4:CD8 ratios in the peripheral blood of ART-treated HIV-1-seropositive individuals are linked to non-AIDS morbidity, immune activation, inflammation and heightened $CD8^+$ T cell activation (43). While there may be a role for specific subsets of $CD8^+$ T-lymphocytes influencing AM behavior in the lung, we found no elevated CD38 expression on BAL T-lymphocytes and no effect on apoptosis or bacterial killing when we explored the influence of activated $CD8^+$ T-lymphocytes on MDM responses to pneumococci *in vitro*. Thus these suggest that global changes in $CD8^+$ T-lymphocytes are a biomarker of intermittent low-level viral replication but do not directly mediate the inhibition of macrophage apoptosis-associated bacterial killing.

We also found evidence for ongoing viral replication in the lungs of some ART-treated individuals by either directly detecting viral RNA, p24 in AM or gp120 in BAL samples. These results add to the observation that potentially replication-competent virus persists in lung AM despite long-term ART (44) and extend reports of detectable gp120 in histological lung specimens of virally suppressed individuals (45). This study measured a snapshot of viral RNA and gp120 and was not powered to detect a relationship between these markers of viral replication and the BAL lymphocyte count or CD4:CD8 ratio. However, the persistence of altered BAL CD4: CD8 T-cell ratios are more likely to be a function of cumulative periods of episodic HIV replication in the lung with normalization of this ratio requiring sustained suppression of viral replication, as described in the peripheral blood (24).

We have been able to demonstrate that recombinant gp120 is sufficient to recapitulate the impairment in delayed phase pneumococcal killing related to HIV-1 infection. HIV-1 envelope (gp120) has been shown to be necessary for macrophage resistance to apoptosis acutely after a single cycle of replication with X4- or R5-tropic HIV-1 (13) while gp120 when disassociated from virus, is sufficient to influence macrophage function and apoptosis resistance (14, 46, 47). Importantly, we observed this effect at concentrations of gp120 similar both to those we found in the BAL and commensurate with those described in other anatomical compartments in HIV-1-seropositive individuals (46).

As with HIV_{BaL}, we observed a failure of gp120 treated MDM to down regulate Mcl-1. Mcl-1 is regulated by ubiquitination and proteasomal degradation (9). Consistent with the paucity of transcriptional changes involving apoptosis regulators in AM from ART-treated HIV-1 donors, we found that gp120 altered post-translational modification of Mcl-1 through reduced ubiquitination in association with upregulation of the DUB USP9X. Thus while Mcl-1 transcriptional upregulation is an immediate intrinsic response to HIV-1 infection (13) we propose that in the context of pneumococcal challenge gp120 mediates the anti-apoptotic phenotype on bystander macrophages through reduced ubiquitination, and the resultant loss of proteasomal degradation of Mcl-1 (10).

gp120 treatment also induced basal mROS but prevented further generation of mROS in response to caspase 3/7 activation following pneumococcal challenge. When we interrogated the bioenergetic response of MDM we observed a switch to glycolytic metabolism following pneumococcal challenge in keeping with a greater reliance on glycolytic metabolism during innate immune responses associated with classical activation in macrophages. We also observed increased proton leak which is predicted to enhance mROS generation since under these conditions complex I is inhibited by caspase activation (27). However, both the uplift in proton leak and loss of mitochondrial inner membrane potential were diminished by gp120. Taken together these results indicate that despite raised baseline levels gp120 reduces caspase-induction of mROS, a critical microbicidal effector (20, 27, 28).

In conclusion, our findings suggest specific defects in the late phase of pneumococcal killing by AM contribute to the sustained increase in susceptibility to pneumococcal disease in PLWH. Furthermore, despite long-term ART, we find evidence of viral replication resulting in release of gp120 in the lungs associated with HIV-1. Through Mcl-1 mediated inhibition of apoptosis, gp120 reduces caspase-dependent induction of mROS and its important microbicidal effects (20). Significantly the inhibition of apoptosis was not part of a global shift in transcriptional networks regulating cell viability but arose in response to impairment of a critical post-translational pathway that regulates macrophage viability. Since the pathway involves ubiquitination of Mcl-1, and is associated with a critical Mcl-1 deubiquitinase USP9X (25), this pathway merits investigation as a potential therapeutic target.

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Study Approval

Healthy donors gave written consent before donating blood for PBMC as approved by the South Sheffield Research Ethics Committee (07/Q2305/7).

HIV-1 seropositive and HIV-seronegative volunteers from the HIV clinics or staff of Sheffield Teaching Hospitals (STH) & the University of Sheffield, Sheffield, UK, gave written informed consent for bronchoalveolar lavage as approved by the NRES Committee Yorkshire & The Humber - South Yorkshire (11/YH/0217).

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Figure legends

Figure 1 HIV-1$_{BaL}$ infection is associated with reduced apoptosis-associated pneumococcal killing by macrophages.

Representative photomicrographs of human monocyte-derived macrophages (MDM) challenged with HIV-1$_{BaL}$ or sham virus and stained for the presence of p24 (blue) (A). Scale bar = 50 μm. Sham or HIV-1$_{BaL}$ MDM were challenged with *S. pneumoniae* (D39) for 4 h (B) or 20 h (C), and lysed to determine the log colony forming units (CFU)/ml, n=15, *=$p<0.05$, paired Student's t-test. Alternatively MDM were challenged with D39 or mock infected (MI) for 16 h and caspase 3/7 luminescence measured (D), n=11, *=$p<0.05$, paired Student's t-test, or for 20 h and the percentage of apoptotic nuclei (E) or cells per high per field (hpf) estimated (F), both n=14, ***=$p<0.001$, *=$p<0.05$, 2 way ANOVA. Additionally cells were challenged for 20 h then lysed and western blot performed for estimation of Mcl-1 (G) and densitometry performed (H), or challenged for 16 h and stained with Mitotracker to estimate mitochondrial density with relative fluorescence units (RFU) (I) or with MitoSOX to estimate fold induction of mitochondrial reactive oxygen species (mROS) vs. sham infection (J), both n=5, **p=<0.01, *=p<0.05, 2 way ANOVA.

Figure 2 People living with HIV have impaired alveolar macrophage apoptosis associated killing of pneumococci.

Alveolar macrophages (AM) from ART treated HIV-1^{+} (ART) or control donors were challenged with *S. pneumoniae* (D39) for 4 h (A) n=8/12 or 20 h (B) n=7/12 and numbers of viable intracellular bacteria determined, *=p<0.05, unpaired Student's t-test. Alternatively HIV-1-seropositive or control AM were exposed to D39 or mock infected (MI) for 16 h and caspase 3/7 activity measured (C), n=5/11, *=p<0.05, unpaired Student's t-test or for 20 h and nuclear features of apoptosis recorded (D) or cell numbers assessed (E) both n=8/14, **=p<0.01, ***=p<0.001, 2 way ANOVA. Nuclear features of apoptosis in AM were determined separately from HIV-1-seropositive donors who had used non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) exclusively as the third ART agent (F), n= 7/6. HIV-1_{BaL} or sham-virus exposed monocyte-derived macrophages (MDM) were challenged D39 for 20 h and apoptosis assessed by nuclear morphology. The value for the HIV-1_{BaL} apoptosis increment was subtracted from the value for the sham-virus exposed MDM increment to calculate the Δ% apoptosis and plotted against the percentage of p24^{+} positive MDM, measured by immunohistochemistry n=13 (G).

Figure 3 People living with HIV have altered T-lymphocyte numbers in the lung associated with markers of viral replication.

Bronchoalveolar lavage (BAL) cells were isolated from HIV-1-seronegative (control n=10) and ART-naive HIV-1-seropositive (naïve, n=3) or ART-treated HIV-1-seropositive (ART n=14) donors and the percentage lymphocytes determined from cytospins (A). Flow cytometry was used to estimate the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ BAL lymphocytes for controls (n=6) and ART-treated (n=11) and the mean ratio of $CD4^+$:$CD8^+$ lymphocytes calculated for each (3.79 ± 0.76 and 1.16 ± 0.15 respectively)(B-D and supplemental figure 2), **=p<0.01, Mann Whitney test, or the expression of CD38 on $CD3^+CD8^+$ BAL lymphocytes (controls n=5, ART n= 9)(E). The ratio correlated to levels of AM apoptosis (n=15)(F), ** p<0.01, Pearson. AM from ART donors were stained with anti-p24 and XGal conjugated secondary antibodies (G). The photomicrograph demonstrates blue p24 positive AM and is representative of photomicrographs from 3 donors, scale bar = 50 μm.

Figure 4 gp120 modifies mitochondrial ROS production following pneumococcal challenge and impairs bacterial killing.

gp120 was measured by sandwich ELISA in the bronchoalveolar lavage (BAL) fluid from 11 ART treated HIV-1 seropositive donors and peripheral blood $CD4^+$ counts were compared in HIV-1-seropositive donors with and without detectable gp120 in the BAL (A), n=5/6, **=p<0.01, Mann Whitney test. Monocyte-derived macrophages (MDM) were treated with 10ng/mL gp120 or media then challenged with *S. pneumoniae* (D39) and viable intracellular bacteria (cfu) were estimated after 4 h (B) and 20 h (C), n=15, *=p<0.05, paired Student's t-test, or nuclear features of apoptosis estimated after 20 h incubation and compared with mock infection (MI)(D), n=8, *=p<0.05, 2 way ANOVA.

Alternatively MDM were treated with 100ng/mL gp120 or media then challenged with D39 or mock infected (MI) for 16 h before quantifying caspase 3/7 activity (E), n=7, *=p<0.05, paired Student's t-test, mitochondrial reactive oxygen species (mROS), in the presence or absence of MitoTEMPO (mT) (F), n=4-8, **=p< 0.01 2 way ANOVA, #=p<0.005 Mann Whitney test (vs. no mT), mitochondrial density (G), n=4, loss of mitochondrial inner transmembrane potential ($\Delta\psi_m$), (H), n=3, *=p<0.05, **=p< 0.01, 2 way ANOVA or using a Seahorse XF24 extracellular flux analyzer to measure oxygen consumption rate (OCR) (I) and extracellular acidification rate (ECAR) (K) and calculate maximum OCR (J), basal ECAR (L) and proton leak (M), all n=6, *=p<0.05, **=p< 0.01, ***=p<0.001, ****=p<0.0001, 2 way ANOVA. Oligo (oligomycin A), Rot (rotenone), AntA (antimycin A).

Figure 5 mROS dependent intracellular pneumococcal killing in macrophages is inhibited by gp120 treatment.

Monocyte-derived macrophages (MDM) were treated with 100ng/mL gp120 or media in the presence of vehicle or MitoTEMPO (mT) then challenged with *S. pneumoniae* (D39) at multiplicity of infection 10 (A) or 100 (B) and viable intracellular bacteria (cfu) were estimated after 4 h and 20 h, n=5 (A), or 20 h n= 6 (B) ****=p<0.0001, *=p<0.05 vs 20 h control, 1 way ANOVA.

Figure 6 gp120 modulates post-translational regulation of Mcl-1 in MDM following pneumococcal challenge.

Monocyte-derived macrophages (MDM) were treated with 100ng/mL gp120 or media then challenged with *S. pneumoniae* (D39) or mock infected (MI) before lysing cells at 20 h and performing Western blots to estimate Mcl-1 (A-B) or lysing cells at 16 h and performing ubiquitin pull-down followed by western blotting for Mcl-1 or total ubiquitinated proteins (C-D). Alternatively cells were lysed at 20 h and blotted for USP9X (E-F). In each case a representative western blot is depicted with the result of densitometry performed on three separate western blots with data shown as fold change in band density compared with mock infected control MDM after adjustment for any fold change in loading control, *=p<0.05, **=p<0.01, 2 way ANOVA.

Tables

Table 1 Healthy and HIV-1 seropositive alveolar macrophage donors

	HIV-1 on ART	HIV-1 ART-NAÏVE	CONTROL
		n or mean ± SEM	
Age (years)	42.4 ± 2.4	41.7 ± 5.3	40.8 ± 2.7
Sex			
Male	8	3	8
Female	6	0	4
Ethnicity			
White	9	3	9
Black African	4	0	3
other	1	0	0
Nadir CD4 (cells/mm^3)	213 ± 26	587 ± 105	n/a
CD4 (cells/mm^3)	643 ± 51	672 ± 176	n/a
CD4:CD8	0.83 ± 0.07	0.66 ± 0.003	n/a
plasma HIV-1 RNA ($\log_{10}$ copies/mL)	undetectable	4.43 ± 3.84	n/a
3^{rd} ART agent			
PI	6	n/a	n/a
NNRTI	7	n/a	n/a
Mixed / other regimen	1	n/a	n/a
Duration (months)	75 (43-108)*		

* median with interquartile range, PI = Protease Inhibitor, NNRTI = Non-Nucleoside Reverse Transcriptase Inhibitor