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1 **Title Page**

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

4 Paul J. Collini^{1,6*}, Martin A. Bewley¹, Mohamed Mohasin¹, Helen M. Marriott¹, Robert F.
5 Miller², Anna-Maria Geretti³, Apostolos Beloukas³, Athanasios Papadimitropoulos³,
6 Robert C. Read⁴, Mahdad Noursadeghi⁵, David H. Dockrell^{1,6,7}

7
8 1 The Florey Institute for Host-Pathogen Interactions and Department of Infection,
9 Immunity & Cardiovascular Disease, University of Sheffield Medical School, Sheffield, UK

10 2 Research Department of Infection and Population Health, Institute of Epidemiology &
11 Health Care, Faculty of Population Health Sciences, University College London, London,
12 UK

13 3. Department of Clinical Infection, Microbiology and Immunology (CIMI), Institute of
14 Infection and Global Health (IGH), University of Liverpool, Liverpool, UK

15 4. Academic Unit of Clinical and Experimental Sciences, University of Southampton and
16 NIHR Southampton Biomedical Research Centre, Southampton, UK

17 5. Division of Infection & Immunity, Faculty of Medical Sciences, University College
18 London, London, UK

19 6. Academic Directorate of Communicable Diseases and Specialised Medicine, Sheffield
20 Teaching Hospitals NHS Foundation Trust, Sheffield, UK

21 7. MRC/UoE Centre for Inflammation Research, The University of Edinburgh, Edinburgh,
22 UK

23
24 ***Corresponding Author:** Paul Collini, Department of Infection, Immunity and
25 Cardiovascular Disease, The University of Sheffield Medical School, Beech Hill Rd,

1 Sheffield, S10 2RX, UK Phone: +44 (0) 114 215 9522 Fax: +44 (0) 114 271 1863 Email:
2 p.collini@sheffield.ac.uk ORCID identifier: 0000-0001-6696-6826

3 **Author contributions**

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

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23
24 **Running Title: gp120 impairs pneumococcal response in HIV lung**

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7 **results 1161**

8 **discussion 1582**

9 **references 47**

10

11 **figures 6 , tables 1,**

12

13 **At a glance summary:**

14 **Scientific Knowledge on the Subject:**

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

17 **What This Study Adds to the Field:**

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

23

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

1 **Structured Abstract**

2
3 **Rationale**

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

7 **Objectives**

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

11 **Methods**

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

21 **Measurements and Main Results**

22 HIV-1_{BaL} infection impaired apoptosis, induction of mROS and pneumococcal
23 killing by MDM. Apoptosis-associated pneumococcal killing was also reduced in
24 AM from ART treated HIV-1-seropositive donors. BAL fluid from these
25 individuals demonstrated persistent lung CD8⁺ T-cell lymphocytosis, and gp120
26 or HIV-1 RNA was also detected. Despite this, transcriptional activity in AM

1 freshly isolated from PLWH was broadly similar to healthy volunteers. Instead,
2 gp120 phenocopied the defect in pneumococcal killing in healthy MDM through
3 post-translational modification of Mcl-1, preventing apoptosis induction, caspase
4 activation and increased mROS generation. Moreover gp120 also inhibited mROS
5 dependent pneumococcal killing in MDM.

6 **Conclusions.**

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

9 **Abstract Word Count 241**

10 **Introduction**

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

19 The capacity of healthy human tissue macrophages to destroy extracellular
20 bacteria through internalization and phagolysosomal killing is finite (6) and AM
21 need to engage a second, delayed microbicidal strategy involving apoptosis-
22 associated killing to eliminate residual viable intracellular pneumococci, which
23 involves combinations of reactive oxygen species (ROS) and nitric oxide (NO) (3,
24 7-9). The apoptotic program is regulated by the anti-apoptotic Bcl-2 protein Mcl-

1 1 and induction of a mitochondrial apoptosis pathway (10). Apoptosis-
2 associated killing enhances clearance of pneumococci, limits tissue invasion and
3 downregulates the inflammatory response in the lung (10, 11). Importantly, HIV-
4 1 is associated with an anti-apoptotic gene expression profile in monocytes *in*
5 *vivo* and promotes macrophage resistance to apoptosis, which contributes to
6 these cells constituting a viral reservoir for HIV-1 (12-15).

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

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

18 **Materials and Methods**

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

21 **Bacteria, Virus and Infections**

22 Opsonized, type 2 *S. pneumoniae* (D39 strain, NCTC7466) were used for infection
23 of macrophages at a multiplicity of infection of 10 unless otherwise stated, as
24 described (10). In some infections autologous peripheral blood lymphocytes
25 (PBL), or HIV-1_{LAI/IIIIB} envelope glycoprotein gp120 (NIBSC, UK) at 10-100 ng/mL

1 were added to MDM. HIV-1_{BaL} (NIH AIDS Reagent Program,) was propagated in
2 PBL, then MDM and purified before cell inoculation. Infection rates were
3 measured by intracellular p24 staining as described (18).

4 **Volunteers**

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

10 **Cell isolation and culture**

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

16 **Western blot**

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

19 **Flow Cytometry**

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

24 **Microscopy**

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

4 **Caspase activation**

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

8 **Quantification of gp120**

9 BAL supernatants were concentrated using 50k Amicon Ultra-filters (Merck
10 Millipore) and gp120 quantified with human monoclonal anti-gp120 antibodies
11 (14E, 17B and EH21), using recombinant gp120 (HIV-1_{LAI/IIIIB}) for standards, by
12 ELISA, as described (19).

13 **Metabolic measurements**

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

17 **RT-PCR Array**

18 AM gene expression was measured after 48 h with a custom made RT² Profiler
19 PCR Array (SABiosciences) using QPCR.

20 **Ultra-sensitive detection of HIV-1 RNA in BAL**

21 BAL HIV-1 RNA was quantified using a modified version of the Abbott Real-Time
22 HIV-1 assay (Maidenhead, UK), following ultracentrifugation similarly to
23 methods in plasma samples (21). After confirming no inhibition, sensitivity was
24 determined at 1-2 copies per mL by spiking acellular HIV-negative BAL with
25 World Health Organization 3rd International HIV-1 RNA Standard (NIBSC, UK).

1 **Statistics**

2 Results are recorded as mean and SEM unless stated. Sample sizes were
3 informed by standard errors obtained from similar assays in prior publications
4 (10, 20). Analysis was performed with tests, as outlined in the figure legends,
5 using Prism 6.0 software (GraphPad Inc.) and significance defined as $p < 0.05$.
6 Decisions on use of parametric or non-parametric tests were informed by the
7 distribution of the data.

10 **Results**

11 **HIV-1 inhibits delayed pneumococcal killing by macrophages**

12 To examine whether HIV-1 influences macrophage killing of pneumococci we
13 infected MDM with HIV-1_{BaL}, an M-tropic strain of HIV-1 (18) or sham virus
14 (Figure 1A) and then, after adjusting for cell numbers, challenged MDM with
15 pneumococci. The numbers of viable intracellular bacteria in MDM 4 h post
16 bacterial challenge, which are the net result of opsonic phagocytosis and
17 phagolysosomal killing (4), were unaltered by HIV-1_{BaL} (Figure 1B). By contrast,
18 20 h after pneumococcal challenge the intracellular bacterial load was higher in
19 HIV-1_{BaL} MDM (Figure 1C). When we examined engagement of the MDM
20 apoptotic programme we found caspase 3/7 activation, development of
21 apoptotic nuclei and loss of cell numbers following pneumococcal challenge
22 were significantly reduced by HIV-1_{BaL} compared to sham infection (Figure 1D-
23 F). Mcl-1 was down-regulated in sham virus exposed MDM but levels were
24 preserved in HIV-1_{BaL} MDM (Figure 1G-H). Despite comparable mitochondrial
25 density HIV-1_{BaL} MDM had elevated production of mROS after mock-infection

1 but, unlike sham virus exposed MDM, failed to upregulate mROS after
2 pneumococcal challenge (Figure 1I-J). Overall these findings support a specific
3 deficit in the delayed apoptosis-associated phase of pneumococcal killing in HIV-
4 _{1BaL} MDM.

6 **Impaired apoptosis-associated pneumococcal killing in alveolar** 7 **macrophages from HIV-1-seropositive individuals treated with ART.**

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

22 **Activation status of AM from HIV-1-seropositive individuals treated with** 23 **ART is similar to healthy volunteers.**

1 We next investigated if steady state expression of representative genes
2 associated with apoptosis and polarization was altered in AM from our donor
3 groups. Using quantitative PCR arrays we found that while there was an overall
4 trend towards downregulation of gene expression in AM from ART treated HIV-
5 1-seropositive individuals compared with healthy controls, no consistent
6 differences in the expression of these genes was observed (supplemental Figure
7 E1). Furthermore, representative markers of macrophage polarization states
8 CD80 (M1), CD163, CD206 and CD200r (M2) also showed no significant
9 alteration in surface expression in AM from HIV-1-seropositive individuals on
10 ART (supplemental Figure E2).

11

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

15 As pulmonary T-lymphocytes influence macrophage-mediated responses to
16 pneumococci in the airway (22), we next sought evidence of alterations to T-
17 lymphocyte numbers in the airway of the asymptomatic HIV-1-seropositive
18 individuals on ART that might link HIV indirectly to the observed AM phenotype.

19 We first analyzed the BAL cell content and included 3 ART-naive HIV-1-
20 seropositive individuals. Both ART-naïve individuals and those receiving ART
21 had increased lymphocyte numbers in BAL fluid (Figure 3A). Compared to
22 healthy controls, ART-treated HIV-1-seropositive individuals also had a lower
23 percentage of CD4⁺ T-lymphocytes yet a higher proportion of CD8⁺ T-
24 lymphocytes and lower CD4⁺:CD8⁺ T-lymphocyte ratio (Figure 3B-D, Table 1 and
25 supplemental Figure E2). In addition, we found that the ratio of CD4⁺:CD8⁺ T-

1 lymphocytes in BAL correlated with the induction of AM apoptosis, following
2 pneumococcal challenge (Figure 3F). We next explored whether T-lymphocyte
3 CD38 expression, a marker of immune activation in HIV-1 that correlates with
4 viral load (23), was increased in the ART-treated HIV-1-seropositive donors.
5 However, CD8⁺ T-lymphocytes showed no difference in CD38 expression (Figure
6 3E). We also tested whether *in vitro* activated, autologous CD8⁺ T-cells, could
7 alter MDM engagement of apoptosis-associated killing but found no modulation
8 of MDM viability, apoptosis or intracellular bacterial survival (supplemental
9 Figure E3A-C).

10 The CD4:CD8 ratio in ART-treated HIV-1-seropositive individuals is inversely
11 related to the size of the HIV-1 reservoir in the peripheral blood (24). Therefore
12 we considered an alternative possibility that BAL CD4:CD8 ratio was a marker of
13 persistent HIV-1 replication in the lung; we detected HIV-1 p24 in AM cultures
14 from 2/2 ART-naïve and 3/10 ART-treated HIV-1-seropositive donors
15 respectively (Figure 3G). Using ultrasensitive assays HIV-1 RNA was detected at
16 79 copies/mL and 1-4 copies/mL of cell free BAL fluid supernatants from 1/1
17 ART-naïve and 2/13 (15.4%) ART-treated HIV-1-seropositive donors
18 respectively. However, the number of donors with detectable p24 or RNA were
19 too few to determine any correlation between these markers of HIV replication
20 and the BAL CD4:CD8 ratio.

21 22 **gp120 impairs bacterial killing by reducing macrophage susceptibility to** 23 **apoptosis following pneumococcal challenge**

24 We detected HIV-1 envelope glycoprotein (gp120) in a 10–100ng/mL range in
25 BAL fluid from five of 11 (45.5%) of the ART-treated and in one of two ART-

1 naïve HIV-1-seropositive donors tested, and observed that those on ART with
2 detectable gp120 also had significantly lower peripheral blood CD4⁺ counts
3 (Figure 4A). Recombinant gp120 recapitulated the selective deficit in delayed
4 phase pneumococcal killing by MDM (Figure 4B-C) and reduced both numbers of
5 apoptotic nuclei and caspase 3/7 activation following pneumococcal challenge
6 (Figure 4D-E). gp120 was also associated with a baseline increase in mROS,
7 without altering mitochondrial density, but gp120 exposed MDM failed to
8 upregulate mROS after pneumococcal challenge (Figure 4F-G). mROS production
9 was abrogated by MitoTEMPO, a mitochondria-targeted superoxide dismutase
10 mimetic that possesses superoxide and alkyl radical scavenging properties,
11 confirming mitochondria as the source of ROS (Figure 4F).

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

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

20 We next analysed whether abrogation of mROS upregulation, with an mROS
21 inhibitor MitoTEMPO, altered intracellular pneumococcal killing. After
22 challenging MDM with pneumococci we observed no difference in the number of
23 viable intracellular bacteria after 4 h in the presence of gp120 or mitoTEMPO.

24 However, addition of MitoTEMPO to control MDM increased bacterial survival at

1 20 h to the same level seen with gp120, but had no effect on viability in gp120
2 exposed MDM at two distinct multiplicities of infection (Figure 5).

3
4 **gp120 impairs macrophage apoptosis by altering the post-translational**
5 **modification of Mcl-1**

6 gp120 prevented downregulation of Mcl-1 (Figure 6A-B) and reduced
7 ubiquitination of Mcl-1 after pneumococcal challenge (Figure 6C-D).

8 Ubiquitination of Mcl-1 is tightly regulated and ubiquitination is reversed by the
9 de-ubiquitinase (DUB) USP9X (25). We detected decreased expression of USP9X
10 following pneumococcal challenge in control MDM but treatment with gp120
11 abrogated this response (Figure 6E-F).

12
13
14 **Discussion**

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

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

11

12 Caspase 3 activation promotes release of mROS by inhibiting the mitochondrial
13 electron transport complex I and has been identified as a requirement for the
14 increment of mROS generation that is required to mediate apoptosis-associated
15 killing of intracellular pneumococci (20, 27). The failure of HIV-1_{BaL} infected
16 MDM to increase mROS production over baseline following pneumococcal
17 challenge resulted in pneumococcal survival, similar to recent observations in
18 AM from COPD patients (20). In contrast to the requirement for a late increment
19 in mROS to achieve optimal intracellular killing, chronic baseline elevation of
20 mROS, following HIV-1 or gp120 exposure, does not seem to enhance
21 intracellular bacterial killing. Consistent with this an inhibitor of mROS had no
22 impact on early intracellular bacterial viability at 4 h. COPD AM also show
23 chronic baseline elevation of intracellular mROS but no enhancement of early
24 intracellular bacterial killing (20). To play a role in intracellular killing mROS
25 needs to be generated in proximity to bacteria in phagolysosomes (20, 28) and

1 be produced at levels above baseline following caspase 3 activation to
2 overwhelm anti-oxidant systems (20). In COPD there is not only reduced
3 caspase 3/7 activation but also an altered balance between mROS generation
4 and superoxide dismutase (SOD) 2 expression, which suggests increased ability
5 to neutralize baseline mROS. Recent observations show gp120 also upregulates
6 SOD in microglia (29). It is noteworthy that, like COPD, HIV-1 has been
7 associated with chronic increases in oxidative stress in mononuclear phagocytes,
8 despite antiretroviral therapy (30, 31), and adaptations to this in both conditions
9 are predicted to impair the capacity to generate a microbicidal response.

10
11 HIV-1 infects and replicates in macrophages and, while establishing a long-lived
12 cellular viral reservoir (15), induces resistance to apoptosis (12, 26). Our finding
13 that HIV-1 infection is linked to intrinsic impairments in macrophage apoptotic
14 responses is supported by previous studies with *Mycobacterium tuberculosis*
15 (32), but to the best of our knowledge ours is the first report of impaired killing
16 of pneumococci or any other acute extracellular bacterial infection. Crucially, we
17 have confirmed our findings in clinically relevant AM from aviraemic HIV-1-
18 seropositive individuals.

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

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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

1 the peripheral blood of ART-treated HIV-1-seropositive individuals are linked to
2 non-AIDS morbidity, immune activation, inflammation and heightened CD8⁺ T
3 cell activation (43). While there may be a role for specific subsets of CD8⁺ T-
4 lymphocytes influencing AM behavior in the lung, we found no elevated CD38
5 expression on BAL T-lymphocytes and no effect on apoptosis or bacterial killing
6 when we explored the influence of activated CD8⁺ T-lymphocytes on MDM
7 responses to pneumococci *in vitro*. Thus these suggest that global changes in
8 CD8⁺ T-lymphocytes are a biomarker of intermittent low-level viral replication
9 but do not directly mediate the inhibition of macrophage apoptosis-associated
10 bacterial killing.

11
12 We also found evidence for ongoing viral replication in the lungs of some ART-
13 treated individuals by either directly detecting viral RNA, p24 in AM or gp120 in
14 BAL samples. These results add to the observation that potentially replication-
15 competent virus persists in lung AM despite long-term ART (44) and extend
16 reports of detectable gp120 in histological lung specimens of virally suppressed
17 individuals (45). This study measured a snapshot of viral RNA and gp120 and
18 was not powered to detect a relationship between these markers of viral
19 replication and the BAL lymphocyte count or CD4:CD8 ratio. However, the
20 persistence of altered BAL CD4: CD8 T-cell ratios are more likely to be a function
21 of cumulative periods of episodic HIV replication in the lung with normalization
22 of this ratio requiring sustained suppression of viral replication, as described in
23 the peripheral blood (24).

24

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

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

22
23 gp120 treatment also induced basal mROS but prevented further generation of
24 mROS in response to caspase 3/7 activation following pneumococcal challenge.
25 When we interrogated the bioenergetic response of MDM we observed a switch

1 to glycolytic metabolism following pneumococcal challenge in keeping with a
2 greater reliance on glycolytic metabolism during innate immune responses
3 associated with classical activation in macrophages. We also observed increased
4 proton leak which is predicted to enhance mROS generation since under these
5 conditions complex I is inhibited by caspase activation (27). However, both the
6 uplift in proton leak and loss of mitochondrial inner membrane potential were
7 diminished by gp120. Taken together these results indicate that despite raised
8 baseline levels gp120 reduces caspase-induction of mROS, a critical microbicidal
9 effector (20, 27, 28).

10
11 In conclusion, our findings suggest specific defects in the late phase of
12 pneumococcal killing by AM contribute to the sustained increase in susceptibility
13 to pneumococcal disease in PLWH. Furthermore, despite long-term ART, we find
14 evidence of viral replication resulting in release of gp120 in the lungs associated
15 with HIV-1. Through Mcl-1 mediated inhibition of apoptosis, gp120 reduces
16 caspase-dependent induction of mROS and its important microbicidal effects
17 (20). Significantly the inhibition of apoptosis was not part of a global shift in
18 transcriptional networks regulating cell viability but arose in response to
19 impairment of a critical post-translational pathway that regulates macrophage
20 viability. Since the pathway involves ubiquitination of Mcl-1, and is associated
21 with a critical Mcl-1 deubiquitinase USP9X (25), this pathway merits
22 investigation as a potential therapeutic target.

23 24 25 **Acknowledgements**

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6

7 **Study Approval**

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

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

14

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7

8 **Figure legends**

9 **Figure 1 HIV-1_{BaL} infection is associated with reduced apoptosis-associated** 10 **pneumococcal killing by macrophages.**

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

1 fold induction of mitochondrial reactive oxygen species (mROS) vs. sham
2 infection (J), both n=5, **p<0.01, *=p<0.05, 2 way ANOVA.

3
4 **Figure 2 People living with HIV have impaired alveolar macrophage**
5 **apoptosis associated killing of pneumococci.**

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

23
24 **Figure 3 People living with HIV have altered T-lymphocyte numbers in the**
25 **lung associated with markers of viral replication.**

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

14

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

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

1 Alternatively MDM were treated with 100ng/mL gp120 or media then
2 challenged with D39 or mock infected (MI) for 16 h before quantifying caspase
3 3/7 activity (E), n=7, *=p<0.05, paired Student's t-test, mitochondrial reactive
4 oxygen species (mROS), in the presence or absence of MitoTEMPO (mT) (F), n=4-
5 8, **=p< 0.01 2 way ANOVA, #=p<0.005 Mann Whitney test (vs. no mT),
6 mitochondrial density (G), n=4, loss of mitochondrial inner transmembrane
7 potential ($\Delta\psi_m$), (H), n=3, *=p<0.05, **=p< 0.01, 2 way ANOVA or using a
8 Seahorse XF24 extracellular flux analyzer to measure oxygen consumption rate
9 (OCR) (I) and extracellular acidification rate (ECAR) (K) and calculate maximum
10 OCR (J), basal ECAR (L) and proton leak (M), all n=6, *=p<0.05, **=p< 0.01,
11 ***=p<0.001, ****=p<0.0001, 2 way ANOVA. Oligo (oligomycin A), Rot
12 (rotenone), AntA (antimycin A).

13

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

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

21

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

24 Monocyte-derived macrophages (MDM) were treated with 100ng/mL gp120 or
25 media then challenged with *S. pneumoniae* (D39) or mock infected (MI) before

1 lysing cells at 20 h and performing Western blots to estimate Mcl-1 (A-B) or
2 lysing cells at 16 h and performing ubiquitin pull-down followed by western
3 blotting for Mcl-1 or total ubiquitinated proteins (C-D). Alternatively cells were
4 lysed at 20 h and blotted for USP9X (E-F). In each case a representative western
5 blot is depicted with the result of densitometry performed on three separate
6 western blots with data shown as fold change in band density compared with
7 mock infected control MDM after adjustment for any fold change in loading
8 control, *=p<0.05, **=p<0.01, 2 way ANOVA.

9
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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) 3 rd ART agent	undetectable	4.43 ± 3.84	n/a
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)*		

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

6

