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Small alveolar macrophages are infected preferentially by HIV and exhibit impaired phagocytic function

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HIV-1-infected persons are at higher risk of lower respiratory tract infections than HIV-1-uninfected individuals. This suggests strongly that HIV-infected persons have specific impairment of pulmonary immune responses, but current understanding of how HIV alters pulmonary immunity is incomplete. Alveolar macrophages (AMs), comprising small and large macrophages, are major effectors of innate immunity in the lung. We postulated that HIV-1 impairs pulmonary innate immunity through impairment of AM physiological functions. AMs were obtained by bronchoalveolar lavage from healthy, asymptomatic, antiretroviral therapy-naive HIV-1-infected and HIV-1-uninfected adults. We used novel assays to detect *in vivo* HIV-infected AMs and to assess AM functions based on the HIV infection status of individual cells. We show that HIV has differential effects on key AM physiological functions, whereby small AMs are infected preferentially by the virus, resulting in selective impairment of phagocytic function. In contrast, HIV has a more generalized effect on AM proteolysis, which does not require direct viral infection. These findings provide new insights into how HIV alters pulmonary innate immunity and the phenotype of AMs that harbors the virus. They underscore the need to clear this HIV reservoir to improve pulmonary immunity and reduce the high incidence of lower respiratory tract infections in HIV-1-infected individuals.

INTRODUCTION

HIV infects a variety of different cell types that have crucial roles in host immunity.^{1–3} CD4⁺ T lymphocytes are the main host cell in chronic HIV infection and the effects of HIV on adaptive immunity have been well documented.^{4,5} HIV infection of other cell types such as macrophages, monocytes, and circulating dendritic cells has, however, been less well studied,⁶ mainly owing to the shortage of reliable methods for detecting *in vivo* HIV-infected cells within these cell populations.

Alveolar macrophages (AMs) are the most abundant phagocytes and major effectors of innate immunity in the alveolar space in the lung.⁷ Recent reports suggest that two macrophage populations, small and large, exist in the alveolar space.^{8,9} AMs perform a variety of important innate functions, including phagocytosis, superoxide burst, and proteolysis.¹⁰

Impaired AM function in smokers is associated with increased risk of pulmonary infections and is implicated in the pathogenesis of chronic obstructive pulmonary disease.^{11,12} These observations highlight the importance of AMs in defence against respiratory pathogens.

Infection with HIV increases the risk of lower respiratory tract infections.^{13,14} AMs are susceptible to HIV infection because they express on their surface CD4, CCR5, and CXCR4 receptors, which mediate HIV entry into cells.¹⁵ Previous approaches have investigated the effect of HIV on AM function at a population level¹⁶ or have used *in vitro* macrophage infection models.¹⁷ Consequently, data on the effects of HIV on the physiological functions of AMs are conflicting. We and others have previously reported unimpaired phagocytic ability of AMs in HIV-infected individuals,^{18–20} but other studies have documented a reduction in the phagocytic capacity of

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Received 6 August 2013; accepted 27 December 2013; published online 29 January 2014. doi:10.1038/mi.2013.127

macrophages.^{21–24} Similarly, although some groups have reported normal killing of bacteria by primary macrophages from HIV-infected individuals,²⁵ others have shown reduced killing capacity of AMs in HIV-infected individuals.^{26,27} Most of these studies assessed only a single AM function and none of them related HIV infection with alterations in function at the level of the individual cell.

To advance current understanding of the direct effect of HIV on AM physiological functions, we developed novel flow cytometry-based assays to detect HIV-infected macrophages by fluorescence *in situ* hybridization (FISH) and to measure macrophage phagocytic capacity, phagosomal superoxide burst, and proteolysis at single-cell level using reporter beads. We conducted a prospective cross-sectional study in healthy, asymptomatic HIV-1-infected and HIV-1-uninfected adults to identify specific defects in AM antimicrobial functions that may predispose HIV-1-infected individuals to lower respiratory tract infections.

RESULTS

Clinical characteristics

Between July 2011 and March 2013, we recruited and performed bronchoalveolar lavage (BAL) on 34 healthy, asymptomatic, and antiretroviral therapy-naïve HIV-1-infected and 45 healthy HIV-1-uninfected adult volunteers (Table 1). Participants were predominantly men (67%) with a mean age of 31 years (range 20–59). Three HIV-1-infected (8.8%) and nine HIV-1-uninfected (20%) participants were smokers. The median peripheral blood CD4+ T-lymphocyte counts were 399 cells μl^{-1} (interquartile range (IQR) = 270–573) and 623 cells μl^{-1} (IQR = 536–723) for HIV-1-infected and HIV-1-uninfected individuals, respectively.

Characterization of AMs

Whole BAL cells from participants were analyzed by flow cytometry. On the basis of their forward scatter and side scatter characteristics and labeling with the macrophage marker, anti-CD206 (for mannose receptor), and the T-lymphocyte marker, anti-CD3 (for T-cell receptor), we defined macrophage and lymphocyte populations, and identified distinct small and large AM subpopulations both in HIV-1-uninfected and HIV-1-infected individuals (Figure 1a,b,c) as previously reported by others.^{8,9} We found no significant difference in the proportions of these cells between HIV-1-uninfected and HIV-1-infected persons (median, small AMs = 32.32% (IQR = 23.5–43.8%)

vs. 30.25% (IQR = 24.8–35.0%), $P > 0.05$; large AMs = 69.6% (IQR = 65.0–75.2%) vs. 67.7% (IQR = 56.2–76.5%); Figure 1d).

To confirm that the small AMs were not inflammatory monocytes or dendritic cells, we measured the proportion and magnitude of expression of monocyte, macrophage, and dendritic cell markers, including the lipopolysaccharide co-receptor (CD14), Fc γ RIII (CD16), mannose receptor (CD206), transferrin receptor (CD71), major histocompatibility complex class II (HLA-DR), integrin- α X (CD11c) and interleukin-3 receptor α -chain (CD123). We found that small AMs expressed predominantly the classical macrophage markers CD71 and CD206, and the pattern of expression was similar to that of large AMs. The proportion and magnitude of expression of the classical monocyte and dendritic cell markers CD14 and CD123, respectively, by small AMs was low (Figure 2a,b). However, there were significant differences in the proportions of surface marker expression by small and large AMs: CD206 (median = 58.0% (IQR = 36.5–78.3%) vs. 85.3% (IQR = 61.6–96.7%), $P = 0.03$), CD71 (median = 82.3% (IQR = 48.3–89.3%) vs. 97.1% (IQR = 85.6–99.0%), $P = 0.03$), and CD16 (median = 51.9% (IQR = 21.8–70.5%) vs. 66.1% (IQR = 32.9–92.1%), $P = 0.03$). There were no significant differences in expression of CD14 and HLA-DR between small and large AMs (Figure 2a). We also found that the magnitude of CD206 expression by small AMs was significantly lower compared with large AMs (median log geometric mean fluorescence intensity = 1.56 (IQR = 1.36–1.70) vs. 1.86 (IQR = 1.72–2.10), $P = 0.03$), but was not significantly different for the other markers evaluated (Figure 2b).

HIV preferentially infects small AMs

To determine the frequency of HIV-infected AMs in BAL from asymptomatic HIV-infected individuals, we used a novel flow cytometry-based FISH assay developed by our group to detect HIV-infected cells. We validated the assay for label specificity using uninfected human monocyte-derived macrophages and human monocyte-derived macrophages infected *in vitro* with the macrophage-tropic HIV-1 strain BaL (Figure 3a). We then used the FISH assay to determine the relative distribution of HIV in BAL macrophages and lymphocytes from six participants with chronic HIV-1 infection by flow cytometric analysis of whole BAL cells, and gating on the macrophage and lymphocyte populations, respectively. The identity of T cells was confirmed by labeling with anti-CD3

Table 1 Characteristics of subjects enrolled in the study

	HIV-1-uninfected adults $n = 45$	HIV-1-infected adults $n = 34$	P -values
Sex, female:male	4:21	14:11	0.0002 ^a
Age, mean years (range)	38 (20–59)	31 (20–51)	0.9672
CD4 count, median cells μl^{-1} (range)	623 (536–723)	399 (270–573)	<0.0001
HIV plasma VL, copies ml^{-1} (range)	NA	21 113 (5803–91 323)	
WHO stage	NA	Stage 1	

NA, not applicable; VL, viral load; WHO, World Health Organisation.

^aFisher's exact test.

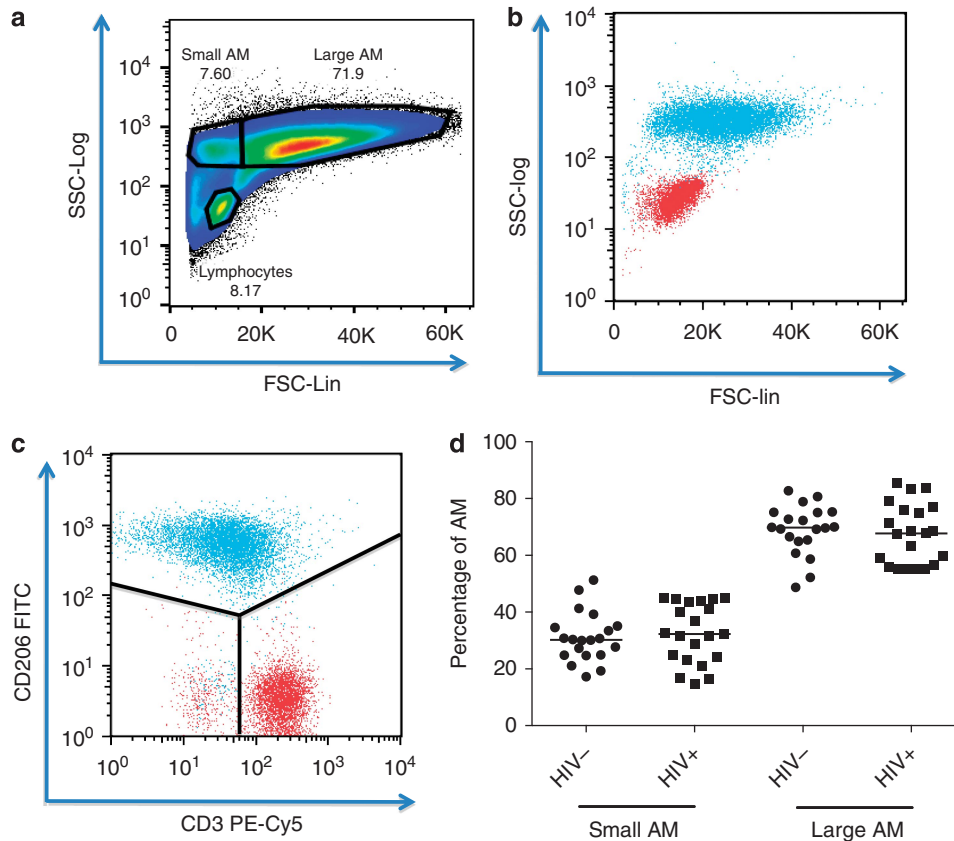


Figure 1 Definition of cell populations in bronchoalveolar lavage (BAL) by flow cytometry. The major cell populations in whole BAL were defined initially by their forward scatter (FSC) and side scatter (SSC) characteristics. The identities of the cell populations were then confirmed by demonstrating the gated cell populations labeled with anti-CD206 (macrophage marker) and anti-CD3 (T-lymphocyte marker), respectively. **(a)** Representative pseudo-color plot showing the gating strategy for macrophage and lymphocyte populations. **(b)** Representative dot plot showing the major cell populations in whole BAL (macrophages = blue and lymphocytes = red). **(c)** Labeling of gated cell populations with macrophage and T-lymphocyte markers, and **(d)** the proportion of small and large alveolar macrophages (AMs) in BAL from HIV-1-infected and HIV-1-uninfected individuals. Data were analyzed using the Mann–Whitney *U*-test; black horizontal bars represent medians (HIV⁻, *n* = 19; HIV⁺, *n* = 22).

antibody (**Figure 3b**). In all six individuals, we detected HIV-infected AMs and T cells (**Figure 3b**); the percentage of HIV-infected cells was higher among AMs than among T cells (median = 1.55% (IQR = 0.64–3.45%) vs. 0.13% (IQR = 0.04–0.59%), *P* = 0.03; **Figure 3c**). However, the frequency of HIV-infected T cells was consistent with what has been reported previously.²⁸

Analysis of adherent BAL cells showed that small AMs constituted the majority of HIV-infected macrophages in BAL (**Figure 4a**) and ~1% of adherent AMs from participants with chronic HIV infection were HIV infected (median = 1.0% (IQR = 0.5–1.3%); **Figure 4b**). We used adherent cells specifically because they consisted of a relatively pure and enriched population of AMs that were used in all subsequent phagosomal function assays. In addition, analysis of whole BAL cells from a subset of five participants revealed a higher frequency of HIV-infected AMs than in adherent BAL cells, suggesting that HIV-infected cells adhere less well to plastic (mean ± s.e.m., whole BAL 3.7% ± 1.3 vs. adherent BAL 1.3% ± 0.3; *P* = 0.06; **Figure 5a,b**). However, as with adherent cells, the proportion of HIV-infected AMs in

whole BAL was higher among the small compared with that among the large AM population (**Figure 5a**). Furthermore, HIV-infected AM showed markedly reduced expression of CD206 (median fluorescence intensity (MFI) = 3.4 (IQR = 2.4–69.2) vs. 87.6 (IQR = 42.2–223.3), *P* = 0.03), HLA-DR (MFI = 27.4 (IQR = 9.4–80.1) vs. 202.5 (IQR = 10.0–236.3), *P* = 0.03), and CD71 (MFI = 6.2 (IQR = 4.7–9.2) vs. 48.9 (IQR = 36.7–49.6), *P* = 0.002), but similar levels of CD45 (MFI = 70.7 (IQR = 46.4–247.3) vs. 70.8 (IQR = 41.3–284.0), compared with HIV-uninfected AMs (**Figure 6a,b**). Reduced expression of CD206 and HLA-DR by HIV-infected human myeloid cells has been reported previously^{22,29} and may represent mechanisms by which HIV alters host innate immunity and accessory cell functions.

Comparison of phagocyte function between small and large AMs

Following identification of small and large AM populations, we investigated whether there were functional differences between them by assessing the ability of these cells to internalize Alexa 405-labeled, IgG-coated reporter beads. We found that the

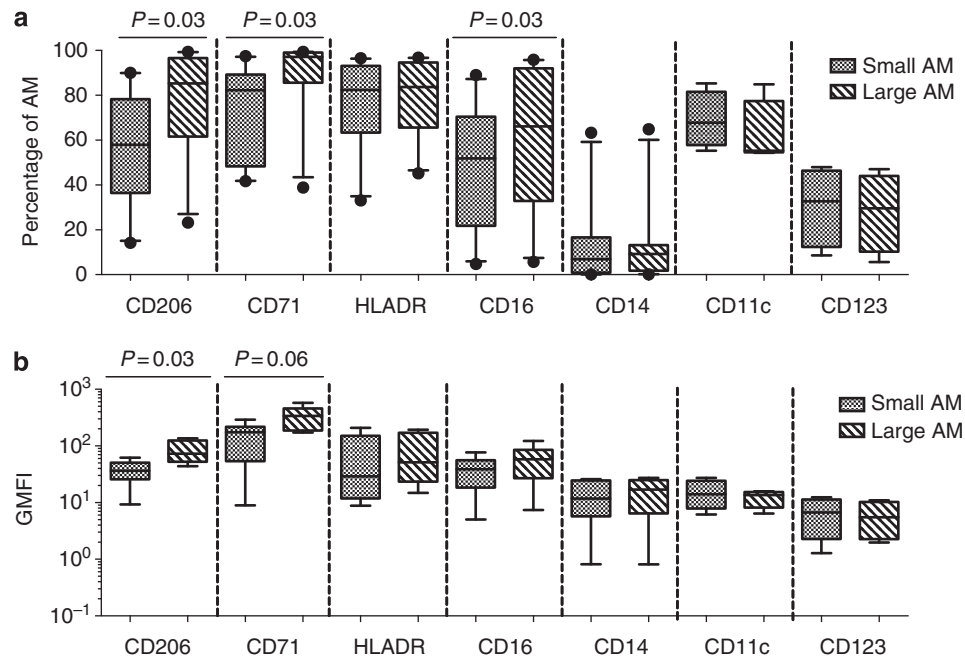


Figure 2 Surface marker expression by small and large alveolar macrophages (AMs) from healthy HIV-1-uninfected participants. Bronchoalveolar lavage (BAL) cells were stained with anti-CD206 fluorescein isothiocyanate (FITC), anti-CD71 PE-Cy5, anti-HLA-DR Alexa fluor 700, anti-CD16 PE, anti-CD14 PE-Cy7, anti-CD11c PE, and anti-CD123 PE-Cy5, and analyzed by flow cytometry. (a) Proportions of small and large AMs expressing these surface markers. (b) The magnitude of surface marker expression by large and small AMs as measured by the geometric mean fluorescence intensity. Data were analyzed using the Wilcoxon matched-pairs signed-ranked test; black horizontal bars represent medians (HIV -, $n=10$).

proportion of large AMs that internalized reporter beads was significantly higher than that of small AMs, both in HIV-1-uninfected and HIV-1-infected persons (HIV-uninfected persons: large AM median = 70.2% (IQR = 58.4–74.0) vs. small AM median = 55.1% (IQR = 41.8–65.3), $P < 0.0001$; HIV-infected persons: large AM median = 65.8% (IQR = 59.6–71.9) vs. small AM median = 58.9% (IQR = 49.1–68.9), $P < 0.0001$; **Figure 7a**). We found no significant differences in phagosomal superoxide burst activity between small and large AMs from HIV-1-infected and HIV-1-uninfected persons (**Figure 7b**). In contrast, we found lower phagosomal bulk proteolytic activity in small compared with large AMs from HIV-1-uninfected persons (mean activity index = 0.16 (95% confidence interval (95% CI) = 0.1–0.2) vs. 0.18 (95% CI = 0.2–0.2), $P = 0.002$), but this was not the case in HIV-infected persons (**Figure 7c**). These data show that compared with large AMs, small AMs from both HIV-1-infected and HIV-1-uninfected individuals have reduced phagocytic capacity. The data also suggest that infection with HIV-1 may impair phagosomal bulk proteolytic activity in both small and large AMs, and this may explain the lack of a significant difference in the magnitude of proteolytic function between these two AM populations in HIV-infected individuals.

Comparison of phagocyte function based on participant HIV status and AM size

Next, we assessed the association between the HIV status of participants and AM function at both the total macrophage population level and as separate populations of small and large

AMs irrespective of the infection status of the AMs. Analysis of total AM populations revealed no significant differences between HIV-1-uninfected and HIV-1-infected participants in the proportions of AMs that internalized reporter beads (median = 65.2% (IQR = 54.8–71.2%) vs. 62.8% (IQR = 56.1–70.2%), $P > 0.05$) and in the magnitude of superoxide burst (mean activity index = 0.10 (95% CI 0.1–0.1) vs. 0.15 (95% CI 0.1–0.2), $P > 0.05$; **Figure 7a,b**). In contrast, we found that the total AM population from HIV-1-infected participants had reduced phagosomal bulk proteolytic activity compared with those from HIV-1-uninfected participants (mean activity index = 0.12 (95% CI 0.1–0.1) vs. 0.17 (95% CI 0.1–0.2), $P = 0.04$; **Figure 7c**). Comparisons of small versus large AM subpopulations revealed reduced superoxide burst and bulk proteolytic activity in large AMs but not in small AMs from HIV-1-infected compared with HIV-1-uninfected participants (mean activity index = 0.09 (95% CI 0.1–0.1) vs. 0.17 (95% CI 0.1–0.2), $P = 0.03$ for superoxide burst (**Figure 7b**) and mean activity index = 0.13 (95% CI 0.1–0.2) vs. 0.18 (95% CI 0.2–0.2), $P = 0.05$ for bulk proteolysis (**Figure 7c**)). These data suggest that HIV-1 is associated with impaired phagosomal activities within the large AM population but not the small AM population from HIV-infected individuals.

Comparison of phagocyte function based on the size and HIV infection status of individual AM

The previous analysis did not discriminate between HIV-infected and HIV-uninfected AMs. To determine whether the impact of HIV on AM function in HIV-1-infected individuals

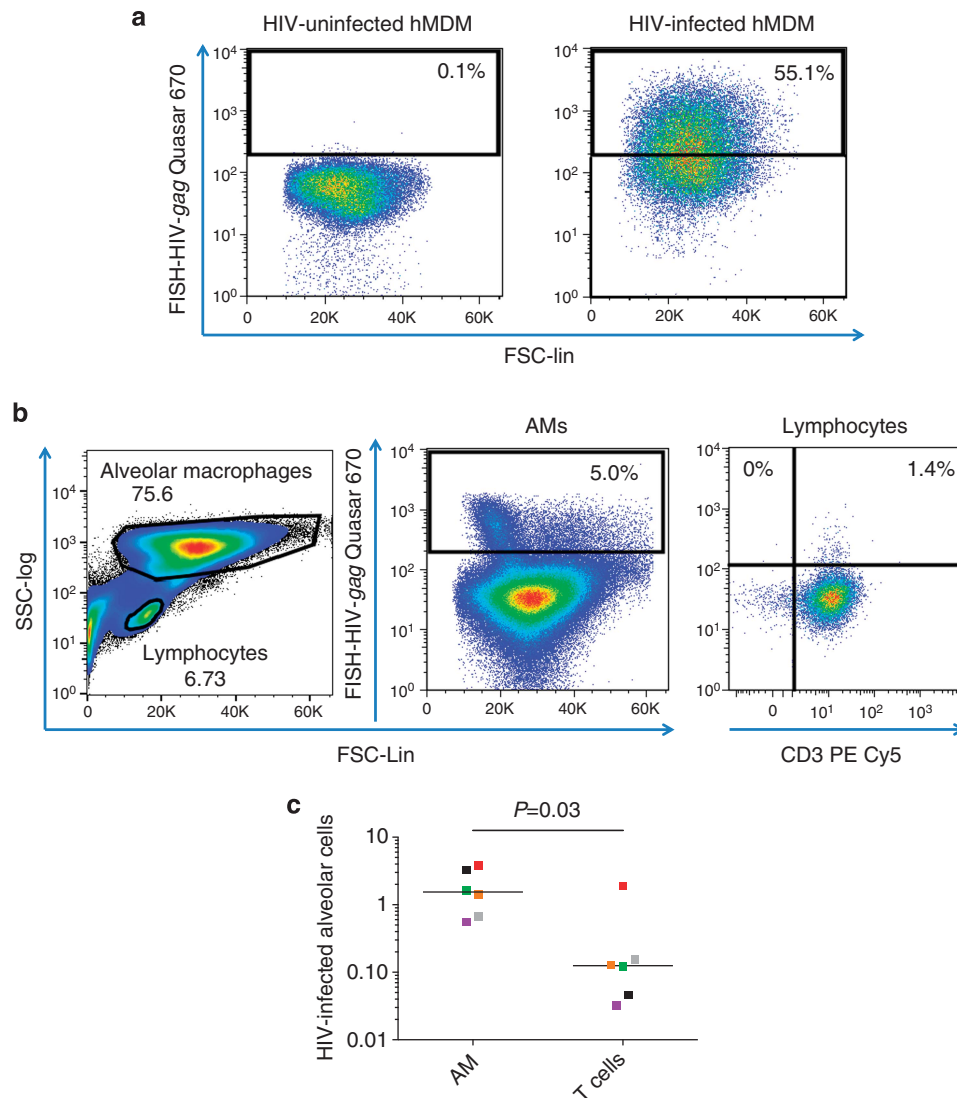


Figure 3 Detection of HIV-infected human cells by fluorescence *in situ* hybridization (FISH). The presence of HIV mRNA in human monocyte-derived macrophages (hMDMs) infected experimentally with the macrophage-tropic strain BaL and in bronchoalveolar lavage (BAL) cells isolated from healthy HIV-infected individuals was detected by flow cytometry using FISH probes against HIV-*gag* mRNA. **(a)** Representative pseudo-color plots from experiments to validate the FISH assay for label specificity using uninfected hMDMs and hMDMs infected *in vitro* with HIV. **(b)** Whole BAL cells were colabeled with HIV-*gag* Quasar 670 FISH probes and anti-CD3 antibody, and analyzed by flow cytometry. Alveolar macrophages (AMs) and lymphocyte populations were identified by their forward scatter (FSC) and side scatter (SSC) characteristic properties. T cells were confirmed by CD3 positivity. **(c)** Detection of HIV-infected cells in AMs and T cells in whole BAL from the same individual (color coded). Data were analyzed using the Wilcoxon matched-pairs signed-rank test; black horizontal bars represent medians ($n=6$).

was specific to HIV-infected cells or a property of all AMs isolated from this tissue environment, we assessed the phagocytic capacity and phagosomal functions of AMs based on the HIV infection status of individual cells. First, we compared, irrespective of cell size, the ability of HIV-infected and HIV-uninfected AMs from the same individual, to internalize reporter beads and to perform phagosomal superoxide burst and bulk proteolysis (**Figure 8a**). We found that overall HIV-infected AMs had increased phagocytic capacity compared with HIV-uninfected AMs (median = 72.3% (IQR = 68.5–75.4%) vs. 62.4% (IQR = 56.1–70.0%), $P=0.004$; **Figure 8b**). However, we found no difference in the magnitude of phagosomal superoxide burst (mean activity

index = 0.10 (95% CI = 0.05–0.14) vs. 0.10 (95% CI = 0.06–0.14), $P>0.05$) or bulk proteolytic activity (mean activity index = 0.11 (95% CI = 0.04–0.18) vs. 0.12 (CI = 0.08–0.16), $P>0.05$) between HIV-infected and HIV-uninfected AMs (**Figure 8c,d**).

Second, we assessed the impact of HIV on the phagocytic and phagosomal functions of HIV-infected AMs by cell size. We found that overall, small AMs had reduced phagocytic capacity irrespective of their HIV-infection status compared with large AMs. However, we found that the difference in phagocytic capacity between small and large AMs was greater in HIV-infected AMs (median = 43.0% (IQR = 28.6–58.6) vs. 80.0% (IQR = 76.4–84.5), $P<0.0001$) compared with HIV-

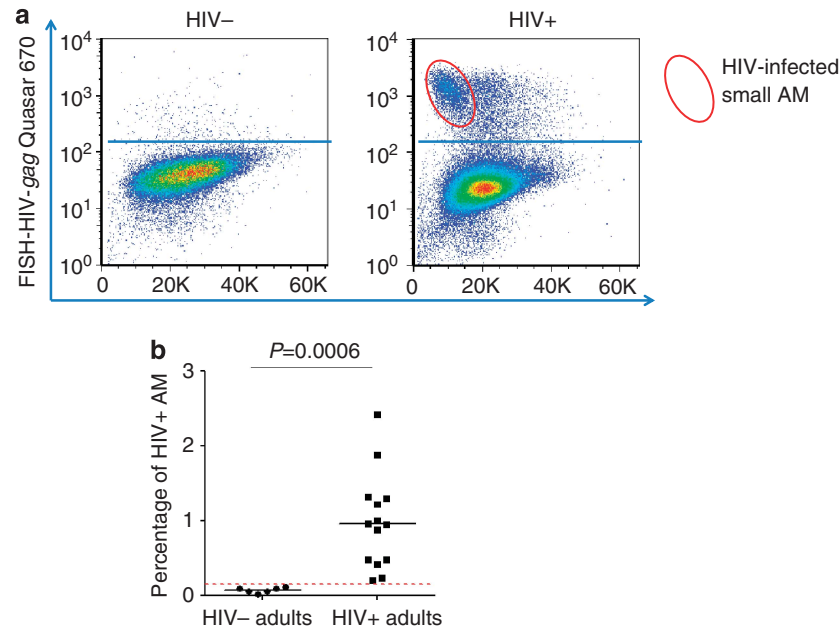


Figure 4 Small alveolar macrophages (AMs) are the dominant macrophage population harboring HIV in the alveolar space. Adherent AMs isolated from bronchoalveolar lavage (BAL) cells of healthy, HIV-infected individuals were stained with fluorescence *in situ* hybridization (FISH) probes against HIV-*gag* mRNA and analyzed by flow cytometry. AMs were identified by their forward scatter (FSC) and side scatter (SSC) characteristic properties and HIV-infected cells were identified by their HIV-*gag* mRNA positivity. (a) Representative flow cytometry pseudo-color plots of adherent AMs from HIV-1-uninfected and HIV-1-infected participants. (b) Proportions of HIV-infected AMs in adherent BAL cells from individuals with chronic HIV infection. Data were analyzed using the Mann-Whitney *U*-test; black horizontal bars represent medians (HIV- , $n = 6$; HIV+ , $n = 14$).

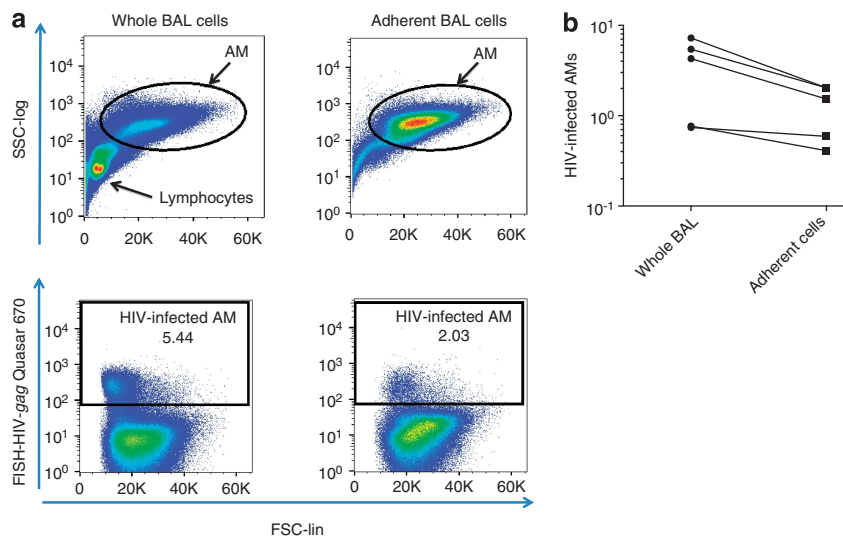


Figure 5 HIV-infected alveolar macrophages (AMs) do not adhere well to plastic. Whole bronchoalveolar lavage (BAL) and adherent BAL cells were stained with HIV-*gag* Quasar 670 fluorescence *in situ* hybridization (FISH) probes and analyzed by flow cytometry. On the basis of forward scatter (FSC) and side scatter (SSC) characteristics of the cells, AMs were identified and the frequency of HIV-infected cells determined. (a) Representative pseudo-color plots showing the gating strategy and the frequency of HIV-infected AMs in whole BAL and adherent BAL cells from the same asymptomatic HIV-infected individual. (b) Comparison of the frequencies of HIV-infected AMs in whole BAL and adherent BAL cells from the same individuals. Data were analyzed using the paired Student's *t*-test ($n = 5$).

uninfected AMs (median = 54.5% (IQR = 46.4–58.0) vs. 65.6% (IQR = 59.1–72.2), $P = 0.003$; **Figure 8b**). We also observed that the phagocytic capacity of HIV-infected small AMs was less than that of HIV-uninfected small AMs, although this did not reach statistical significance (**Figure 8b**). Interestingly, the

phagocytic capacity of HIV-infected large AMs was significantly greater than that of HIV-uninfected large AMs (80.0% (IQR = 76.4–84.5) vs. 65.6% (IQR = 59.1–72.2), $P < 0.0001$). We found no difference in phagosomal superoxide burst or bulk proteolytic activity between small

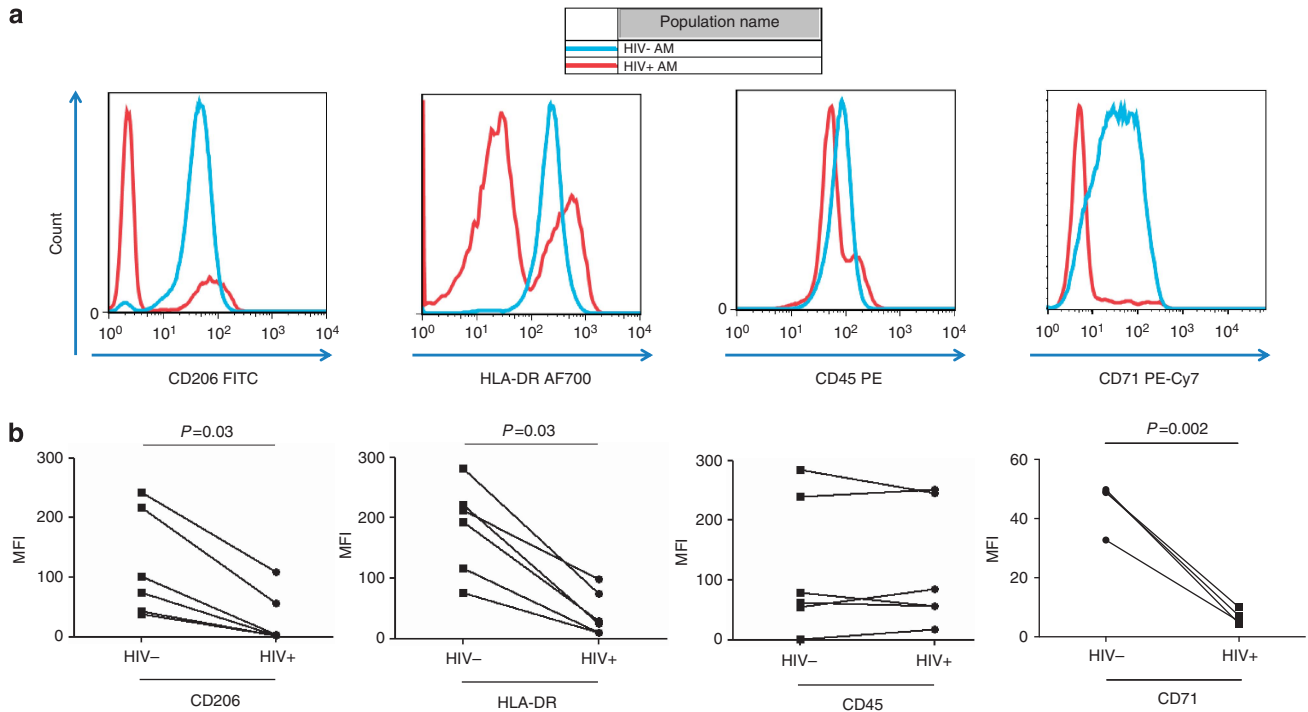


Figure 6 Surface marker expression by HIV-infected alveolar macrophages (AMs) in whole bronchoalveolar lavage (BAL) from HIV-1-infected participants. Whole BAL cells stained with anti-CD206 fluorescein isothiocyanate (FITC), anti-HLA-DR AF700, anti-CD45 PE, and anti-CD71 PE-Cy5 for surface marker expression, and HIV-*gag* Quasar 670 fluorescence *in situ* hybridization (FISH) probes to detect HIV-infected cells were analyzed by flow cytometry. AMs were identified by their forward scatter (FSC) and side scatter (SSC) characteristics and the expression of CD206, HLA-DR, CD45 and CD71 by HIV-infected and HIV-uninfected AMs from the same individual was determined. **(a)** Representative histograms showing expression of CD206, HLA-DR, CD45 and CD71 by HIV-infected and HIV-uninfected AMs. **(b)** Expression of CD206, HLA-DR, CD45 and CD71 by HIV-infected and HIV-uninfected AMs. Data were analyzed by the Wilcoxon matched-pairs signed-ranked test ($n=6$).

and large HIV-infected or HIV-uninfected AMs (**Figure 8c,d**). These findings suggest that HIV has differential effects on AM function whereby HIV-infected small AMs exhibit selective impairment of phagocytic function. In contrast, HIV has a more generalized effect on AM proteolysis, which is not restricted to HIV-infected cells but affects all AMs isolated from this tissue environment.

DISCUSSION

In this study, we assessed the impact of HIV on the physiological functions of AMs, major contributors to innate immunity in the lower respiratory tract. We have shown that small and large AMs are present in BAL from both HIV-1-infected and HIV-1-uninfected individuals, and that in HIV-infected individuals small AMs are the predominant phenotype that harbors HIV.

Small macrophages with monocytic characteristics have previously been identified in sputum and BAL from patients with cystic fibrosis.^{8,9} In addition, it has been shown that non-classical monocytes expressing CD16 in the peripheral blood are permissive to infection and preferentially harbor HIV-1 *in vivo*.^{30,31} We wondered, therefore, whether the population of small, HIV-preferred macrophages we had identified in BAL were inflammatory monocytes. We found that apart from the size difference, small AMs expressed the same array of surface markers typical of macrophages and expressed by the large AMs

such as CD206 and CD71, which are not expressed on inflammatory monocytes.^{32,33} Functionally, however, we have shown that small AMs from both HIV-1-infected and HIV-1-uninfected individuals have reduced phagocytic capacity and bulk proteolytic activity than large AMs. This is consistent with the findings of Lay *et al.*,³⁴ who reported that the phagocytic capacity of airway mature macrophages was significantly greater than airway immature macrophages in healthy participants. We speculate that the functional differences observed could be attributed to the differentiation stage of the AMs, small AMs may be immature macrophages differentiating into large mature AMs, and originate either from peripheral blood monocytes or from progenitor cells within the lung parenchyma.^{35,36}

Only a small proportion of AM in asymptomatic chronically HIV-1-infected adults are infected with HIV. This is consistent with previous studies that reported low frequency of HIV-infected AMs in chronically HIV-infected individuals.^{37,38} We have demonstrated that impaired phagocytosis is specific to HIV-infected small AMs, which also have reduced expression of HLA-DR and CD206, an important pathogen recognition receptor.³⁹ In the current study, we did not determine whether the virus preferentially infects AMs with this surface marker phenotype or whether the induction of this phenotype occurred post infection. However, Koziel *et al.*²² have reported previously that *in vitro* HIV infection of AMs from healthy individuals significantly reduced mannose receptor (CD206)

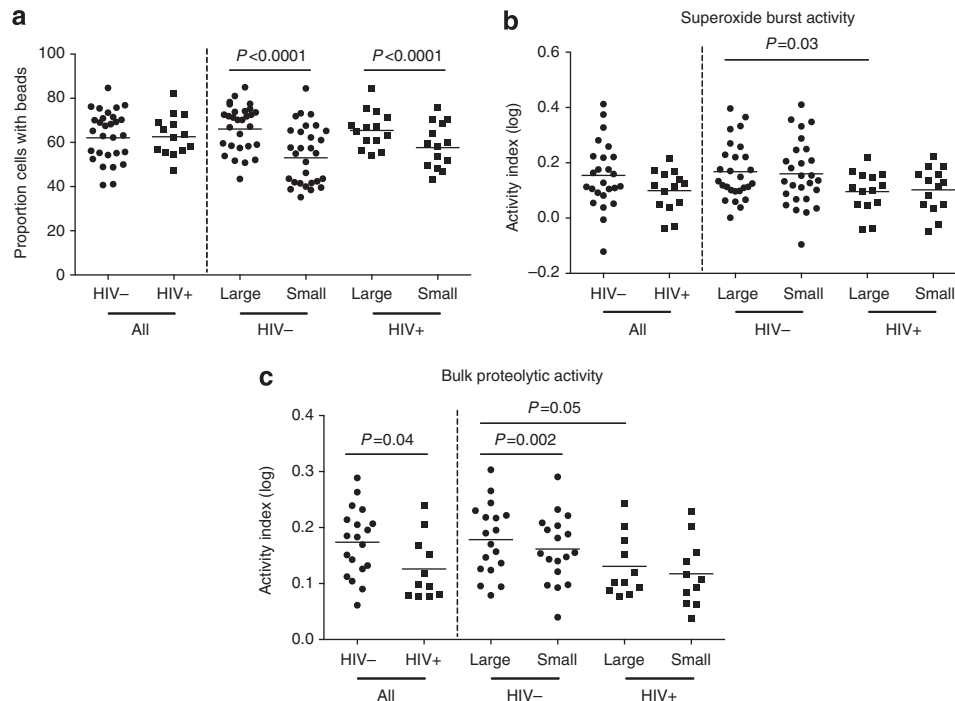


Figure 7 Assessment of alveolar macrophage (AM) function based on the HIV infection status of participants. Adherent AMs were incubated with reporter beads to measure phagocytosis, superoxide burst, and bulk proteolysis, and analyzed by flow cytometry. (a) Phagocytosis of reporter beads by large and small AMs (HIV⁻, *n* = 25; HIV⁺, *n* = 14). (b) Phagosomal superoxide burst activity in AMs from HIV-1-infected compared with HIV-1-uninfected participants (HIV⁻, *n* = 25; HIV⁺, *n* = 14). (c) Phagosomal proteolytic activity in AMs from HIV-1-infected compared with HIV-1-uninfected participants (HIV⁻, *n* = 18; HIV⁺, *n* = 11). The data in panels b and c are expressed as the Activity Index, which is the ratio of relative fluorescence units of the substrate fluorescence divided by the calibration fluorescence to ensure dosage correction for minor variations in bead number. Data were analyzed by Mann–Whitney *U*-test (HIV⁺ vs. HIV⁻) and Wilcoxon matched-pairs signed-ranked test (small vs. large AMs) (a), or unpaired (HIV⁺ vs. HIV⁻) and paired Student's *t*-test (small vs. large AMs) on log-transformed data (b,c); black horizontal bars represent medians (a) and means (b,c).

endocytosis and *Pneumocystis carinii* (*Pneumocystis jirovecii*) binding and phagocytosis compared with HIV-uninfected AMs. These *in vitro* findings suggest that reduced expression of CD206 and HLA-DR by HIV-infected small AMs occurred post infection and may represent potential mechanisms by which HIV alters pulmonary innate immunity and AM physiological and accessory functions.

We have shown that impaired phagosomal proteolytic activity is not restricted to HIV-infected AMs but is a property of all AMs in this tissue environment. Although our data do not address the mechanisms responsible for the reduced proteolytic activity of AMs from HIV-1-infected individuals, Yates *et al.*⁴⁰ reported reduced phagosomal proteolysis following activation of murine bone marrow-derived macrophages by interferon- γ and concluded that activation reduces the early phagosomal hydrolytic capacity of macrophages. Asymptomatic HIV-1-infected individuals have high levels of interferon- γ in the lung⁴¹ and have highly activated AMs compared with HIV-uninfected individuals.⁴² We speculate that the reduced phagosomal proteolytic activity observed in AMs from HIV-1-infected individuals may be due to interferon- γ -induced activation of these cells. Although AMs may not be the dominant antigen-presenting cells in the lung due to low expression of costimulatory molecules such as CD80 and CD86,⁴³ reduced proteolytic function coupled with reduced

HLA-DR expression by HIV-infected AMs would further affect their ability to process and present antigens to T cells, thereby contributing to impaired bronchoalveolar CD4⁺ T-cell responses.^{44,45} It would also limit the immune recognition of HIV-infected AM, which will blunt the effector functions and, potentially, reduce clearance of infected cells.

In this study, we did not determine whether HIV infection of AMs is productive and generates progeny virus. In addition, we did not look directly at the microbial killing behavior of AMs using infectious agents but restricted our preliminary functional analysis to short-term, well-defined and characterized assays such as proteolysis and superoxide burst. This is because we are currently unable to safely perform culture of infectious agents beyond short-term incubations in the clinical laboratories in Malawi. However, we intend to address these important shortfalls in the future and to directly assess the microbial killing behavior of AMs infected *in vitro* with respiratory pathogens such as *Mycobacterium tuberculosis*.

In conclusion, we have shown that HIV affects innate immunity in the lower respiratory tract through impairment of key AM physiological functions. The ability to detect HIV-infected AMs and to assess macrophage function based on the HIV infection status of individual cells has enabled us to establish that impairment of some phagocyte functions such as phagocytosis is selective and predominantly affects

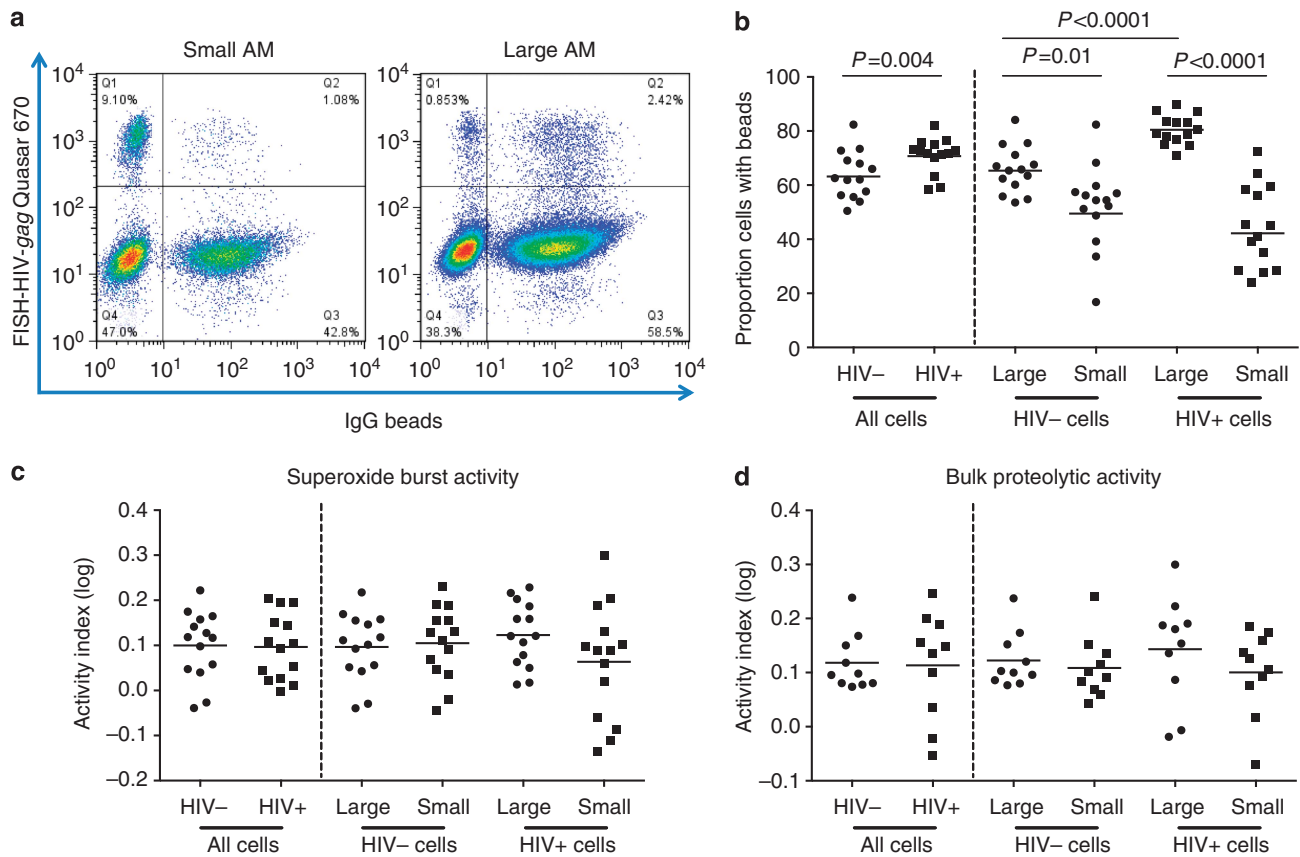


Figure 8 Assessment of alveolar macrophage (AM) function based on the HIV infection status of individual cells. Adherent AMs were incubated with reporter beads to measure phagocytosis, superoxide burst, and bulk proteolysis. The cells were then stained with HIV-*gag* Quasar 670-labeled probes by fluorescence *in situ* hybridization (FISH) to detect HIV-infected AMs and analyzed by flow cytometry. **(a)** Representative flow cytometry pseudo-color plots showing phagocytosis of beads by small and large, HIV-infected, and HIV-uninfected AMs. The plots show four different cell populations: HIV-infected cells without beads (Q1), HIV-infected cells with beads (Q2), HIV-uninfected cells with beads (Q3), and HIV-uninfected cells without beads (Q4). **(b)** Phagocytosis of reporter beads by HIV-infected and HIV-uninfected AMs from the same HIV-infected individuals ($n=14$). **(c,d)** Phagosomal superoxide burst and bulk proteolytic activities, respectively, in HIV-infected compared with HIV-uninfected AMs ($n=10$). The data in panels **c** and **d** are expressed as the Activity Index, which is the ratio of relative fluorescence units of the substrate fluorescence divided by the calibration fluorescence to ensure dosage correction for minor variations in bead number. Data were analyzed using matched-pair one-way analysis of variance with Bonferroni correction; black horizontal bars represent medians (**b**) and means (**c,d**).

HIV-infected small AMs, whereas impairment of other functions such as phagosomal proteolysis is generalized and affects all AMs from HIV-1-infected individuals. AMs are infected with HIV early during the course of HIV infection⁴⁶ and are relatively resistant to the cytopathic effects of HIV, making them suitable reservoirs for the virus.⁴⁷ Therefore, persistent HIV infection of AMs will result in early and protracted impairment of pulmonary innate and adaptive immunity. Impaired AM innate functions may explain, at least in part, the high incidence of lower respiratory tract infections seen in HIV-1-infected individuals. Whether initiation of antiretroviral therapy, particularly using agents that target viral integration within the host genome, reverses this immune defect remains to be determined.

METHODS

Subjects. The study was conducted at the Queen Elizabeth Central Hospital, a large teaching hospital in Blantyre, Malawi. Participants were healthy adults (≥ 18 years) comprising asymptomatic

HIV-1-infected and HIV-1-uninfected volunteers with no clinical evidence of active disease and willing to undergo bronchoscopy specifically for research purposes. All HIV-1-infected participants were antiretroviral therapy naive at the time of recruitment. Exclusion criteria were use of immunosuppressive drugs, anemia ($\text{Hb} < 8 \text{ g dl}^{-1}$) and known or suspected pregnancy. The study was approved by the research ethics committees of the College of Medicine in Malawi, the Liverpool School of Tropical Medicine and Cornell University. All participants provided written informed consent.

Bronchoscopy and processing of BAL. Bronchoscopy and BAL were performed as previously described.^{44,48} Because of limitations in cell numbers, not all the assays were performed on cells obtained from every participant.

Immunophenotyping and enrichment of AMs. Immunophenotyping was performed on 5×10^5 whole BAL cells before any enrichment step. Cells were stained with anti-CD206 FITC, anti-CD3 PE-Cy5, anti-CD16 PE, anti-CD71 PE-Cy5, anti-CD14 PE-Cy7, anti-CD11c PE, anti-CD123 PE-Cy5, and anti-HLADR AlexaFluor700 (all BD Bioscience, Oxford, UK), and acquired on a CyAn ADP 9-Colour flow cytometer (Beckman Coulter, Brea, CA). Data were analyzed using FlowJo software version 7.6.4 (Tree Star, San Carlos, CA). For each

stained sample analyzed, the MFI for each parameter was normalized to its respective unstained control. AMs were enriched from whole BAL cells by adherence to >99% purity as described previously.⁴⁹

FISH assay for detection of HIV-infected AMs. HIV-infected cells were detected by flow cytometry using a modification of the Stellaris RNA FISH assay targeting HIV gag mRNA using a pool of 48 fluorophore-labeled oligonucleotides⁵⁰ (Biosearch Technologies, Novato, CA). Briefly, adherent AMs were fixed in 0.5 ml of 3.7% formaldehyde for 15 min. Fixed cells were washed and permeabilized in 70% ethanol overnight at 4 °C. Cells were then washed in buffer (10% formamide, 20 × saline sodium citrate, and nuclease-free water) and resuspended in hybridization buffer (10% formamide, 20 × saline sodium citrate, 10% dextran sulfate with 1 mg ml⁻¹ *Escherichia coli* tRNA, 200 µg ml⁻¹ RNase-free bovine serum albumin, and 2 mM vanadyl ribonucleoside complex). HIV-1-gag Quasar 670-labeled probes were incubated with the cells for 5 h at 37 °C in the dark. Following hybridization, the cells were washed twice in wash buffer, resuspended in 0.5 ml phosphate buffered saline, and acquired on a flow cytometer. Although the FISH probes have the potential to detect both viral RNA and pro-viral DNA, the denaturation protocol that we used is unlikely to melt duplex DNA. Furthermore, the number of copies of mRNA will greatly exceed that of DNA; hence, the signal is almost exclusively due to the detection of viral mRNA.

Measurement of AM phagocytosis, superoxide burst, and bulk proteolysis. Phagocytosis and enzymatic activities in the phagosomal compartments of AMs were measured using quantitative flow cytometry-based reporter bead assays as reported previously.¹⁰ In brief, the assays exploit silica beads derivatized with a calibration fluorochrome (Alexa 405-SE) and the fluorogenic reporter substrates Oxyburst Green, succinimidyl ester (Molecular Probes, Eugene, OR) for superoxide burst or DQ Green bovine serum albumin (Molecular Probes) for bulk proteolysis. When the beads are internalized by AMs, they gain fluorescence intensity proportional to the degree of activity in the phagosome. The readout is expressed as a ratio of the substrate fluorescence to the calibration fluorescence.

For each assay, 100 µl per well of Oxyburst-SE (superoxide burst) or DQ bovine serum albumin (bulk proteolysis) beads suspension were added to adherent cells in six-well culture plates. Cells were collected at two time points, although the duration of the assays was different. The first time point was 10 min after adding beads to wells. Cells were collected by gentle scrapping into suspension, transferred to a tube containing 0.5 ml of 3.7% formaldehyde fixative, and incubated in the dark at room temperature for 15 min. The plate was re-incubated until the second time point (60 min for superoxide burst or 240 min for proteolysis) when the cells were collected and processed as described above. Fixed cells were washed twice in phosphate buffered saline, resuspended in 0.5 ml phosphate buffered saline, and acquired on a flow cytometer. The readout for phagocytosis was the proportion of cells that had internalized the beads after 60 min. The readout for the superoxide burst and bulk proteolysis assays was the Activity Index, which was calculated by first determining the ratio of MFI of the reporter over calibration flour at 10- and 60-min, or 240-min time points, and then dividing the ratio at 60 or 240 min by ratio at 10 min. To assess AM function based on the HIV infection status of individual cells, reporter bead assays were combined with the FISH assay for detecting HIV-infected cells.

Statistical analyses. Statistical analyses and graphical presentation were done using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Normally distributed data were analyzed using paired or unpaired Student's *t*-test, whereas non-normally distributed data were analyzed using Wilcoxon matched-pairs signed-ranked test (paired data) or Mann-Whitney *U*-test (non-paired data). One-way analysis of variance with Bonferroni correction was used for multiple comparisons on paired data. Results are given as medians with IQRs, except for

Activity Indices and the comparison of frequency of HIV-infected AMs in whole BAL compared with adherent cells, which are given as means with CIs, and mean with s.e.m., respectively. Differences were considered statistically significant when $P < 0.05$.

ACKNOWLEDGMENTS

We thank all study participants, Mrs Malamba, Mrs Kunkeyani, Mrs Kanyandula, and staff of MLW and QECH for their support and co-operation during the study; Marc Beal and Ron Cook of Biosearch Technologies for help in the development of the anti-HIV Stellaris RNA FISH probes. We also thank Professor Stephen Gordon for reading the manuscript. This work was funded by the Wellcome Trust (UK) through an Intermediate Clinical Fellowship number 088696/Z/09/Z awarded to H.C.M., the National Institutes of Health (USA) through grant numbers HL100928 and AI095519 awarded to D.G.R., and the National Commission of Science and Technology-HRCSI, Malawi, through grant number RG-J/2009/KJ/0003 awarded to K.C.J. R.S.H. is supported by Core Funding to the Malawi-Liverpool-Wellcome Trust Clinical Research Programme from the Wellcome Trust.

DISCLOSURE

The authors declared no conflict of interest.

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