HIV-1 Infection Impairs CD16 and CD35 Mediated Opsonophagocytosis of *Mycobacterium tuberculosis* by Human Neutrophils

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Abstract: Using a flow cytometric assay, we investigated neutrophil–*Mycobacterium tuberculosis* opsonophagocytosis and the impact of HIV-1–infected serum on this process. The mean (±SD) percentage of neutrophils internalizing bacilli after 30 minutes incubation was significantly reduced by pretreatment with anti-CD16 (18.2% ± 8.1%, P < 0.001) or anti-CD35 antibody (23.2% ± 10.6%, P < 0.05) versus anti-CD4 controls (29.9% ± 8.1%). Blocking CD88 or CD11a did not affect internalization. Using heat-inactivated serum, maximal internalization was lower using HIV-1–infected serum versus HIV-1–uninfected. Using non–heat-inactivated serum, internalization decreased more rapidly with sequential dilutions of HIV-1–infected versus HIV-1–uninfected serum. CD16 and CD35 are important for neutrophil internalization of *M. tuberculosis*, whereas HIV-1 infection adversely affects opsonophagocytosis.

Key Words: neutrophil, phagocytosis, opsonization, tuberculosis, mycobacteria, HIV


METHODS

Serum Donors and Preparation

For receptor-blocking experiments, serum was prepared from 21 consenting healthy laboratory donors using SST tubes (Becton Dickinson, South Africa), pooled and stored at −80°C. For comparison of serum depending on HIV-1 status, asymptomatic antiretroviral therapy–naïve HIV-1–infected persons with CD4 count <350 × 10⁶/mL were recruited from the Ubuntu HIV clinic, Khayelitsha, South Africa. The HIV-1–uninfected cohort was recruited from the Ubuntu clinic or Khayelitsha Site B Youth Centre among asymptomatic individuals with a recent negative HIV test. HIV testing, viral load, and CD4 count were performed by the South African National Health Laboratory Service. To generate serum, fresh blood was centrifuged at 500g in 15 mL Falcon tubes for 15 minutes before transferring plasma to a new tube in a water bath at 37°C; after platelets had plugged serum was aspirated and stored at −80°C. Donors provided written, informed consent. The study protocol was approved by the University of Cape Town Research Ethics Committee (HREC 545/2010).

Heat inactivation was performed in a water bath at 56°C for 30 minutes.
Isolation of Human Neutrophils

This has been described previously. Briefly, 25 mL blood from healthy laboratory donors were sedimented using 6% dextran, the cell-rich supematant was suspended in 3 mL 55% Percoll and layered onto a discontinuous gradient of 81% Percoll and 70% Percoll. Tubes were centrifuged at 720 × g with no deceleration and granulocytes harvested from the 81%/70% interface before Coulter counting.

Organisms and Labeling

1.5 mL vials of M. tuberculosis-lux (prepared as described8) were defrosted from storage at −80°C and added to 15 mL 7H9/ADC (Becton Dickinson) containing 0.05% Tween 80 (Sigma) and 1 ml/mL hygromycin B (Roche). Organisms were cultured before use to mid-log phase (72 hours). Fluorescein isothiocyanate (FITC) labeling was performed as described on 5 mL of organism culture9; mycobacteria were resuspended in 7H9 medium and then diluted with phosphate-buffered saline to reach the desired inoculum immediately before infection.

Phagocytosis Assay

This assay has been described in detail. During receptor-blocking experiments, the methodology was applied as published, ie, 50 ml of serum (10% final concentration) and 50 ml of FITC-labeled M. tuberculosis at multiplicity of infection (MOI) 1 Colony Forming Unit (CFU): 3 neutrophils were added to 400 ml of neutrophils suspended in RPMI-1640 at a concentration of 1 × 10⁹/ml.

For experiments comparing serum opsonic capacity, the methodology was modified to resemble opsonophagocytosis assays developed for Streptococcus pneumoniae.10 Serum was serially diluted 1:1 with RPMI-1640 during preparation. Neutrophils were resuspended at 2 × 10⁶/ml, and 200 ml of this suspension were added to tubes together with 250 ml prediluted serum and 50 ml organisms. This modification allowed a maximum serum concentration of 50%, although preliminary experiments indicated maximal internalization at 25% final serum concentration (reported herein as “1 in 4”). The next dilution assessed was 1 in 8 for heat-inactivated serum and 1 in 32 for non–heat-inactivated serum (preliminary experiments indicated little reduction in internalization at concentrations between 1 in 8 and 1 in 32 for non–heat-inactivated serum). Dilutions then proceeded 2-fold. To ensure adequate internalization even with heat-inactivated serum, a higher MOI was used of 3 CFU: 1 neutrophil. Each pairwise comparison of an HIV-1–infected and HIV-1–uninfected serum used a single donor’s neutrophils. In addition to the percentage of neutrophils internalizing organisms at each serum concentration, we also calculated a “breakpoint,” the concentration at which internalization fell to less than 50% of that seen at maximum concentration. If 50% of maximum internalization was not reached, then the breakpoint was defined as a 2-fold further dilution than the last dilution tested. We also repeated the analysis excluding those samples which failed to reach breakpoint.

After samples were prepared, they were incubated for 30 minutes on rocking plates at 37°C and then processed at 0°C as described.9 The flow cytometric analysis is summarized in Figure 1A. Results are presented as total percentage of CD66+ neutrophils positive for FITC signal.

Blocking Antibodies

Neutrophils were incubated on ice with azide-free antibodies for 30 minutes before the addition of serum and mycobacteria (volumes determined by preliminary titration experiments): 10 ml anti-CD4 (control), 0.5 ml anti-CD11a, 10 ml anti-CD16, 10 ml anti-CD35, and 0.5 ml anti-CD88 (all from Becton Dickinson).

Statistical Analysis

Two groups were compared using Student’s t test, 3 or more groups were analyzed by 1-way analysis of variance. Statistical analysis was performed using GraphPad Prism v4.0 or later.

RESULTS

CD35 and CD16 Are Important for Internalization of M. tuberculosis by Neutrophils

Preincubation of neutrophils from 9 donors with anti-CD35 or anti-CD16 antibodies reduced internalization of FITC-labeled M. tuberculosis versus anti-CD4 controls in the presence of pooled healthy donor serum (Fig. 1B). The mean ± SD percentage of neutrophils internalizing mycobacteria after 30 minutes incubation was 18.2 ± 8.1% with anti-CD16 antibody (P < 0.001) and 23.2% ± 10.6% with anti-CD35 antibody (P = 0.01) versus 29.9% ± 8.1% in anti-CD4 controls. Incubation with anti-CD11a had no effect (28.9% ± 9.2%). Incubation with antibodies against all receptors suggested a cumulative effect of CD16 and CD35, with a mean ± SD percentage neutrophils internalizing of 13.3 ± 5.4% (P < 0.001 versus controls; Fig. 1B).

Blockade of CD11a or CD88 Does Not Affect Internalization of M. tuberculosis by Neutrophils

Preincubation of neutrophils from 8 donors with anti-CD11a or anti-CD88 antibodies, singly or in combination, did not affect internalization of FITC-labeled M. tuberculosis versus anti-CD4 controls (Fig. 1C). The mean ± SD percentage of neutrophils internalizing mycobacteria was 26.0% ± 9.3% with anti-CD11a antibody, 27.2% ± 8.1% with anti-CD88 antibody, 23.6% ± 10.3% with both antibodies, and 27.0% ± 6.8% in controls (P ≥ 0.18 for all comparisons). In preliminary experiments, there was no effect on phagocytosis even of higher concentrations of these antibodies (anti-CD11a up to 5 μL and anti-CD88 up to 10 μL).

The Opsonic Capacity of Serum From HIV-1–Infected Persons Is Inferior to that of HIV-1–Uninfected Persons

The results above suggest a role for both complement (by CD35) and antibodies (by CD16) in mediating neutrophil phagocytosis of M. tuberculosis. To investigate the impact of
HIV-1 infection on the activity of these serum opsonins, we serially diluted heat-inactivated and non-heat-inactivated serum from 8 antiretroviral-naive HIV-1-infected and HIV-1-uninfected donors for use in the phagocytosis assay. Supplemental Table 1 provides donor demographic details (see Supplemental Digital Content, http://links.lww.com/QAI/A846).

Results using heat-inactivated samples revealed reduced internalization with HIV-1-infected persons’ serum at higher concentrations (mean ± SD percentage of neutrophils internalizing with HIV-1 serum at final concentration 1 in 4: 16.7% ± 13.7% versus non–HIV-1 serum, 32.6% ± 22.6%, P = 0.048; at concentration 1 in 8: 14.6% ± 10.8% versus non–HIV-1 serum, 31.6% ± 21.8%, P = 0.011; Fig. 2A). At lower serum concentrations, there was relatively little internalization and no significant differences between HIV-1-infected and HIV-1-uninfected donors. There was also no difference in “breakpoints” between the 2 groups (Fig. 2C). Of note, maximal internalization in patients with positive interferon gamma release assay results (n = 6) was higher than in those with negative (n = 9) results (36.1% ± 17.5% versus 15.4% ± 18.0%, P = 0.046).

For non-heat-inactivated serum, there was no difference in maximal internalization, but at further dilutions of serum the mean ± SD percentage of neutrophils internalizing organisms was significantly reduced using HIV-1–infected patients’ serum (at final concentration 1 in 32: 40.9% ± 37.0% versus non–HIV-1 serum, 77.0% ± 13.7%, P = 0.016; at concentration 1 in 64: 31.5 ± 37.0% versus 72.3% ± 14.0%, P = 0.010; at concentration 1 in 128: 24.7% ± 30.8% versus 65.1% ± 16.9%, P = 0.016; Fig. 2B). Beyond these concentrations, there was no difference between the 2 groups (although we were unable to assess all donors at these concentrations). Correspondingly, the “breakpoints” in the HIV-1–infected group were seen at significantly higher concentration (mean ± SD for HIV-infected 1 in 120 ± 113 versus HIV-uninfected 1 in 384 ± 274, P = 0.048; Fig. 2C). There remained a significant difference after excluding 2 samples which did not reach breakpoint: mean ± SD for HIV-infected 1 in 120 ± 113 versus HIV-uninfected 1 in 299 ± 105, P = 0.026.

**DISCUSSION**

There is a critical role for neutrophils in the host response to tuberculosis. They are likely to be protective...
against initial infection,1–3 which may be of special importance in people with compromised acquired immune responses such as in HIV-1 infection, but may play a detrimental role in active disease. Nevertheless, it is clear that many bacilli are inside neutrophils during human tuberculosis infection,6 and it is therefore important to study their internalization.

Here, we have first confirmed our own9 and others’7 findings that complement is a critical mediator of neutrophil–M. tuberculosis opsonophagocytosis, demonstrated by a significant reduction in the percentage of neutrophils phagocytosing bacilli with heat inactivation of serum. Correspondingly, blocking CD35 (CR-1) before phagocytosis significantly reduced internalization. By contrast, the C5a receptor CD88 did not seem to play a significant role and nor does CD11a seem to be important in the opsonophagocytosis process.

Despite this apparently major role for complement, we saw the greatest reduction in phagocytosis with blockade of CD16 (Fcγ-Receptor Receptor IIIb). This may represent a previously described phenomenon whereby CD16 ligation is required for subsequent CR3 binding.11 However, it may also suggest an independent role for antibodies. Even using heat-inactivated serum we discovered that, with high MOI and 25% serum concentration, 32.6% ± 22.6% of neutrophils from HIV-1–uninfected donors were positive for FITC signal. There was also higher maximal internalization in participants with evidence of sensitization to M. tuberculosis (positive interferon gamma release assay result, although this may be confounded by HIV status). We have previously demonstrated modest neutrophil phagocytosis of Mycobacterium bovis Bacille Calmette-Guerin (BCG) with 10% heat-inactivated adult human serum but a complete absence of internalization with heat-inactivated fetal calf serum, which presumably lacks antimycobacterial antibodies.9 This corresponds with findings by others, for example greater neutrophil internalization in the presence of heat-inactivated serum versus no serum7 and increased internalization of M. bovis BCG in donor samples after BCG vaccination (presumably due to antibody production).12

Having demonstrated that serum opsonins enhance neutrophil–M. tuberculosis opsonophagocytosis, we assessed the opsonic capacity of serum from antiretroviral therapy–naïve HIV-1–infected and HIV-1–uninfected individuals. Opsonophagocytosis for Candida albicans and S. pneumoniae is impaired by HIV-1 infection,13,14 and we here extend that conclusion to M. tuberculosis. Using heat-inactivated serum,
we observed a reduction in maximal internalization. For non-heat-inactivated serum, there was an earlier reduction in phagocytosis with serial dilution and earlier “breakpoints,” suggesting global impairment of *M. tuberculosis* opsonization. This may relate to ineffective antibody responses. Alternatively, we postulate that the polyclonal hypergamma-globulinemia characteristic of HIV-1 infection may interfere with effective opsonophagocytosis. Interestingly, high doses of IgG administered therapeutically may act through CD16 to reduce CR3 expression. Indeed, we noted in preliminary experiments that incubation with 50% serum yielded consistently lower internalization than with 25% serum whether heat-inactivated or not (*n* = 3 for both groups, statistically significant reduction in non-heat-inactivated group).

An omission from our receptor-blocking experiments may be CD11b/CD18 (CR3), which could underpin our inability to fully explain the apparent effect of complement. Some experiments were performed with a higher CFU:neutrophil MOI to ensure significant internalization with heat-inactivated serum. As described, there are potential issues with this approach including “clumping” of organisms. However, these experiments represented direct comparisons between 1 HIV-1–infected and 1 non–HIV-1–infected serum sample with other conditions standardized (including donor neutrophils and infecting inoculum) which should have avoided systematic bias.

In conclusion, we have delineated some of the critical mediators of opsonophagocytosis of *M. tuberculosis* by human neutrophils and demonstrated an impairment of this process by HIV-1 infection. These findings may inform therapeutic interventions. First, our results demonstrate that HIV-1 infection compromises an early line of defense against tuberculosis; this supports prompt initiation of antiretroviral therapy. Conversely, if neutrophils contribute to pathology in established tuberculosis disease, especially with a “Trojan horse” role, then strategies to block internalization of bacilli should be considered: CD16 or CD35 would be appropriate targets.

REFERENCES


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