

OPEN

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

Nonzwakazi Bangani, BSc,* Justine Nakiwala, BSc,*†‡ Adrian R. Martineau, PhD,§||¶ Robert J. Wilkinson, PhD,*||¶ Katalin A. Wilkinson, PhD,*¶ and David M. Lowe, PhD*||#

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 (\pm SD) percentage of neutrophils internalizing bacilli after 30 minutes incubation was significantly reduced by pretreatment with anti-CD16 ($18.2\% \pm 8.1\%$, $P < 0.001$) or anti-CD35 antibody ($23.2\% \pm 10.6\%$, $P < 0.05$) versus anti-CD4 controls ($29.9\% \pm 8.1\%$). 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

(*J Acquir Immune Defic Syndr* 2016;73:263–267)

BACKGROUND

The host immune response to tuberculosis remains incompletely understood, but neutrophils are increasingly recognized as having important protective and pathogenic roles.^{1–3}

Neutrophils are professional phagocytes which internalize mycobacteria to kill them,² traffic them to distant sites,⁴ or act as immunologically privileged sites allowing bacillary survival or replication.^{5,6} Phagocytosis by neutrophils is improved by opsonization, as has been specifically demonstrated for *M. tuberculosis*.⁷

We therefore sought to interrogate the biology behind opsonization and phagocytosis of *M. tuberculosis* by human neutrophils. First, we investigated which cell surface receptors are important for mediating internalization, focusing on complement receptors (CRs) and Fc- γ receptors that mediate macrophage phagocytosis of tuberculosis bacilli.⁸ Subsequently, to address a clinically relevant question, we compared the opsonization capacity of serum from HIV-1–infected and HIV-1–uninfected persons.

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 $<350 \times 10^6/\text{mL}$ 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.

Received for publication December 4, 2015; accepted May 23, 2016.

From the *Clinical Infectious Diseases Research Initiative, Institute for Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa; †Institute of Tropical Medicine, Antwerp, Belgium; ‡Medical Research Council (MRC)/Uganda Virus Research Institute (UVRI) Uganda Research Unit on AIDS, Entebbe, Uganda; §Blizard Institute, Barts and The London School of Medicine, Queen Mary University of London, London, United Kingdom; ||Department of Medicine, Imperial College London, London, United Kingdom; ¶Mill Hill Laboratory, The Francis Crick Institute, London, United Kingdom; and #Institute of Immunity and Transplantation, University College London, London, United Kingdom.

Supported by Wellcome Trust (104803, 097684, 087754, 084323); MRC (UK) U1175.02.002.00014; European Union (FP7 HEALTH-F3-2012-305578 and FP7-PEOPLE-2011-IRSES); National research Foundation of South Africa (96841).

The authors have no conflicts of interest to disclose.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

Correspondence to: David M. Lowe, PhD, Institute of Immunity and Transplantation, University College London, Royal Free Campus, Pond Street, London NW3 2QG, United Kingdom (e-mail: d.lowe@ucl.ac.uk).

Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved. This is an open access article distributed under the Creative Commons Attribution License 4.0 (CCBY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Isolation of Human Neutrophils

This has been described previously.⁹ Briefly, 25 mL blood from healthy laboratory donors were sedimented using 6% dextran, the cell-rich supernatant was suspended in 3 mL 55% Percoll and layered onto a discontinuous gradient of 81% Percoll and 70% Percoll. Tubes were centrifuged at 720g 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 described⁹) were defrosted from storage at -80°C and added to 15 mL 7H9/ADC (Becton Dickinson) containing 0.05% Tween 80 (Sigma) and 1 mcl/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.⁹ During receptor-blocking experiments, the methodology was applied as published, ie, 50 mcl of serum (10% final concentration) and 50 mcl of FITC-labeled *M. tuberculosis* at multiplicity of infection (MOI) 1 Colony Forming Unit (CFU): 3 neutrophils were added to 400 mcl of neutrophils suspended in RPMI-1640 at a concentration of $1 \times 10^6/\text{mL}$.

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 $2 \times 10^6/\text{mL}$, and 200 mcl of this suspension were added to tubes together with 250 mcl prediluted serum and 50 mcl 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 mcl anti-CD4 (control), 0.5 mcl anti-CD11a, 10 mcl anti-CD16, 10 mcl anti-CD35, and 0.5 mcl 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 \pm SD percentage of neutrophils internalizing mycobacteria after 30 minutes incubation was $18.2\% \pm 8.1\%$ with anti-CD16 antibody ($P < 0.001$) and $23.2\% \pm 10.6\%$ with anti-CD35 antibody ($P = 0.01$) versus $29.9\% \pm 8.1\%$ in anti-CD4 controls. Incubation with anti-CD11a had no effect ($28.9\% \pm 9.2\%$). Incubation with antibodies against all receptors suggested a cumulative effect of CD16 and CD35, with a mean \pm SD percentage neutrophils internalizing of $13.3 \pm 5.4\%$ ($P < 0.001$ 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 \pm SD percentage of neutrophils internalizing mycobacteria was $26.0\% \pm 9.3\%$ with anti-CD11a antibody, $27.25\% \pm 8.1\%$ with anti-CD88 antibody, $23.6\% \pm 10.3\%$ with both antibodies, and $27.0\% \pm 6.8\%$ in controls ($P \geq 0.18$ for all comparisons). In preliminary experiments, there was no effect on phagocytosis even of higher concentrations of these antibodies (anti-CD11a up to $5 \mu\text{L}$ and anti-CD88 up to $10 \mu\text{L}$).

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

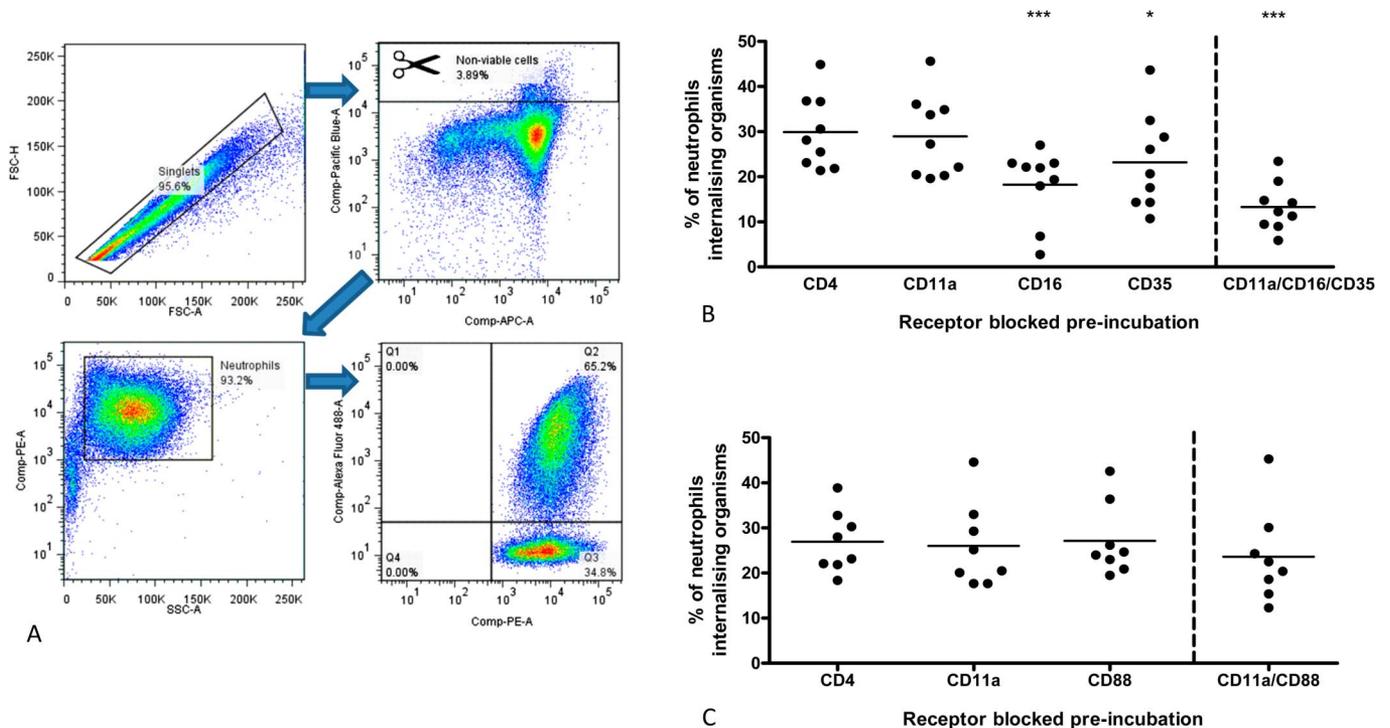


FIGURE 1. The effect of blocking neutrophil surface receptors on the phagocytosis of *M. tuberculosis*. A, Representative plot of flow cytometry analysis. First, singlet signals are gated by Forward Scatter (FSC)-Area (A) versus Height (H). Next, dead cells are excluded with the eFluor450 Viability Dye (Pacific Blue channel). Neutrophils are gated through high CD66a,c,e-PE expression and finally the percentage of neutrophils positive for FITC-labeled *M. tuberculosis* signal is calculated. B, 4×10^5 neutrophils isolated from healthy donors ($n = 9$) were incubated on ice for 30 minutes with antibodies against the receptor(s) indicated before the addition of 10% pooled donor serum and FITC-labeled *M. tuberculosis*-lux at MOI 1 CFU: 3 neutrophils. After 30 minutes incubation, samples were processed at 0°C, stained with eFluor450 Viability Dye and CD66a,c,e-PE and extracellular fluorescence quenched with trypan blue. The y-axis depicts the percentage of viable CD66a,c,e-positive neutrophils, which had internalized organisms (positive for FITC signal). Lines represent mean. C, Samples from 8 healthy donors were processed as in (B) using antibodies against the receptors indicated. Data expressed as in (B). * $P < 0.05$, *** $P < 0.001$ (repeated measures 1-way analysis of variance for individual receptor-blocking conditions, post hoc Bonferroni correction comparing each neutrophil receptor antibody with anti-CD4 control; paired *t* test of “all” condition versus CD4 controls).

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 \pm SD percentage of neutrophils internalizing with HIV-1 serum at final concentration 1 in 4: 16.7% \pm 13.7% versus non-HIV-1 serum, 32.6% \pm 22.6%, $P = 0.048$; at concentration 1 in 8: 14.6% \pm 10.8% versus non-HIV-1 serum, 31.6% \pm 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% \pm 17.5% versus 15.4% \pm 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 \pm SD percentage of neutrophils internalizing organisms was significantly reduced using HIV-1-infected patients’ serum (at final concentration 1 in 32: 40.9% \pm 37.0% versus non-HIV-1 serum, 77.0% \pm 13.7%, $P = 0.016$; at concentration 1 in 64: 31.5 \pm 37.0% versus 72.3% \pm 14.0%, $P = 0.010$; at concentration 1 in 128: 24.7% \pm 30.8% versus 65.1% \pm 16.9%, $P = 0.009$; 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 \pm SD for HIV-infected 1 in 120 \pm 113 versus HIV-uninfected 1 in 384 \pm 274, $P = 0.048$; Fig. 2C). There remained a significant difference after excluding 2 samples which did not reach breakpoint: mean \pm SD for HIV-infected 1 in 120 \pm 113 versus HIV-uninfected 1 in 299 \pm 105, $P = 0.026$.

DISCUSSION

There is a critical role for neutrophils in the host response to tuberculosis. They are likely to be protective

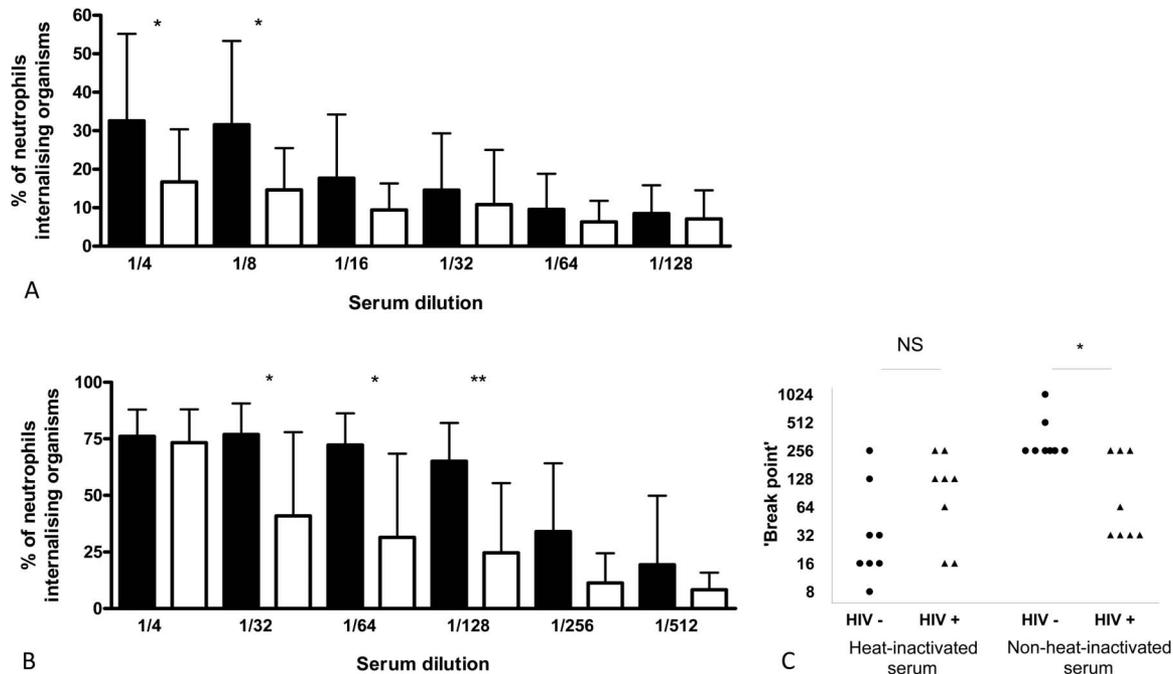


FIGURE 2. The effect of HIV on the opsonizing capacity of serum for neutrophil phagocytosis of *M. tuberculosis*. A, Heat-inactivated serum from HIV-infected donors (white columns) and HIV-uninfected donors (black columns) was serially diluted 2-fold with RPMI-1640 before adding 250 μ L of each concentration to 4×10^5 healthy donor neutrophils and FITC-labeled *M. tuberculosis*-lux at MOI 3 CFU: 1 neutrophil. The final serum concentration is depicted on the x-axis. After 30 minutes incubation, samples were processed at 0°C, stained with Viability Dye and CD66a,c,e-PE and extracellular fluorescence quenched with trypan blue. The y-axis depicts the percentage of viable CD66a,c,e-positive neutrophils which had internalized organisms (positive for FITC signal). Each experiment used a single donor's neutrophils and compared 1 HIV-infected with 1 HIV-uninfected serum sample. $n = 8$, overall ($n = 7$ for 1/8 dilution), column heights represent mean, error bars represent SD. B, Non-heat-inactivated samples from the same donors were processed as in (A). $n = 8$ overall ($n = 7$ for 1/256 dilution, $n = 6$ for 1/512 dilution). Data presented as in (A). C, "Breakpoints" were defined from the results of (A) and (B) as the reciprocal of the serum dilution at which internalization fell to 50% of maximum and are presented for each serum donor/condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (paired t tests).

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.^{3,5} 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 others⁷ 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 (Fc γ -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% \pm 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-naive 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.¹⁵ Alternatively, we postulate that the polyclonal hypergammaglobulinemia 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.¹⁶ 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 approach⁹ 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,^{4,6} then strategies to block internalization of bacilli should be considered: CD16 or CD35 would be appropriate targets.

REFERENCES

- Martineau AR, Newton SM, Wilkinson KA, et al. Neutrophil-mediated innate immune resistance to mycobacteria. *J Clin Invest*. 2007;117:1988–1994.
- Kisich KO, Higgins M, Diamond G, et al. Tumor necrosis factor alpha stimulates killing of Mycobacterium tuberculosis by human neutrophils. *Infect Immun*. 2002;70:4591–4599.
- Lowe DM, Redford PS, Wilkinson RJ, et al. Neutrophils in tuberculosis: friend or foe?. *Trends Immunol*. 2012;33:14–25.
- Abadie V, Badell E, Douillard P, et al. Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood*. 2005;106:1843–1850.
- Yeremeev V, Linge I, Kondratieva T, et al. Neutrophils exacerbate tuberculosis infection in genetically susceptible mice. *Tuberculosis (Edinb)*. 2015;95:447–451.
- Eum SY, Kong JH, Hong MS, et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest*. 2010;137:122–128.
- Majeed M, Perskvist N, Ernst JD, et al. Roles of calcium and annexins in phagocytosis and elimination of an attenuated strain of *Mycobacterium tuberculosis* in human neutrophils. *Microb Pathog*. 1998;24:309–320.
- Restrepo BI, Twahirwa M, Rahbar MH, et al. Phagocytosis via complement or Fc-gamma receptors is compromised in monocytes from type 2 diabetes patients with chronic hyperglycemia. *PLoS One*. 2014;9:e92977.
- Lowe DM, Bangani N, Mehta MR, et al. A novel assay of antimycobacterial activity and phagocytosis by human neutrophils. *Tuberculosis (Edinb)*. 2013;93:167–178.
- Martinez JE, Romero-Steiner S, Pilishvili T, et al. A flow cytometric opsonophagocytic assay for measurement of functional antibodies elicited after vaccination with the 23-valent pneumococcal polysaccharide vaccine. *Clin Diagn Lab Immunol*. 1999;6:581–586.
- Preynat-Seauve O, Villiers CL, Jourdan G, et al. An interaction between CD16 and CR3 enhances iC3b binding to CR3 but is lost during differentiation of monocytes into dendritic cells. *Eur J Immunol*. 2004;34:147–155.
- de Valliere S, Abate G, Blazevic A, et al. Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. *Infect Immun*. 2005;73:6711–6720.
- Takahashi H, Oishi K, Yoshimine H, et al. Decreased serum opsonic activity against *Streptococcus pneumoniae* in human immunodeficiency virus-infected Ugandan adults. *Clin Infect Dis*. 2003;37:1534–1540.
- Tachavanich K, Pattanapanyasat K, Sarasombath S, et al. Opsonophagocytosis and intracellular killing activity of neutrophils in patients with human immunodeficiency virus infection. *Asian Pac J Allergy Immunol*. 1996;14:49–56.
- Yu X, Prados-Rosales R, Jenny-Avital ER, et al. Comparative evaluation of profiles of antibodies to mycobacterial capsular polysaccharides in tuberculosis patients and controls stratified by HIV status. *Clin Vaccine Immunol*. 2012;19:198–208.
- Jang JE, Hidalgo A, Frenette PS. Intravenous immunoglobulins modulate neutrophil activation and vascular injury through Fc-gammaRIII and SHP-1. *Circ Res*. 2012;110:1057–1066.