HIV-1 infection impairs CD16 and CD35 mediated opsonophagocytosis of *M.tuberculosis* by human neutrophils

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Conflicts of Interest and Sources of Funding: The authors declare no conflicts of interest. Funding: 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).

Running title: Neutrophil-TB opsonophagocytosis

Abstract

Using a flow cytometric assay we investigated neutrophil-*M.tuberculosis* opsonophagocytosis and the impact of HIV-1-infected serum on this process. The mean (±SD) percentage of neutrophils internalising bacilli after 30 minutes incubation was significantly reduced by pre-treatment 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 internalisation. Using heat-inactivated serum, maximal internalisation was lower using HIV-1-infected serum versus HIV-1-uninfected. Using non-heat-inactivated serum, internalisation decreased more rapidly with sequential dilutions of HIV-1-infected versus HIV-1-uninfected serum. Conclusions: CD16 and CD35 are important for neutrophil internalisation of *M.tuberculosis* while HIV-1 infection adversely affects opsonophagocytosis.

Key words: Neutrophil, phagocytosis, opsonisation, tuberculosis, mycobacteria, HIV

Background

The host immune response to tuberculosis remains incompletely understood, but neutrophils are increasingly recognised as having important protective and pathogenic roles [1-3].

Neutrophils are professional phagocytes which internalise 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 opsonisation, as has been specifically demonstrated for *M.tuberculosis* [7].

We therefore sought to interrogate the biology behind opsonisation and phagocytosis of *M.tuberculosis* by human neutrophils. First we investigated which cell surface receptors are important for mediating internalisation, focussing on complement receptors and Fc-γ receptors that mediate macrophage phagocytosis of tuberculosis bacilli [8]. Subsequently, to address a clinically relevant question, we compared the opsonisation 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^{o}C. For comparison of serum depending on HIV-1 status, asymptomatic ART-naïve HIV-1-infected persons with CD4 count <350 x10^{6}/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 amongst 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 500 x g in 15ml Falcon tubes for 15 minutes before transferring plasma to a new tube in a water bath at 37^{o}C; after platelets had plugged serum was aspirated and stored at -80^{o}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^{o}C for 30 minutes.

Isolation of human neutrophils

This has been described previously [9]. Briefly, 25ml blood from healthy laboratory donors were sedimented using 6% dextran, the cell-rich supernatant was suspended in 3ml 55% Percoll and layered onto a discontinuous gradient of 81% Percoll and 70% Percoll. Tubes were centrifuged at 720 x g with no deceleration and granulocytes harvested from the 81%/70% interface before Coulter counting.

Organisms and labelling

1.5ml vials of *M.tuberculosis*-lux (prepared as described [9]) were defrosted from storage at -80^{o}C and added to 15ml 7H9/ADC (Becton Dickinson) containing 0.05% Tween 80 (Sigma) and 1mcl/ml hygromycin B (Roche). Organisms were cultured before use to mid-log phase (72 hours). FITC labelling was performed as described on 5ml of organism culture [9];

mycobacteria were resuspended in 7H9 medium and then diluted with PBS 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, i.e. 50mcl of serum (10% final concentration) and 50mcl of FITC-labelled *M.tuberculosis* at multiplicity of infection (MOI) 1 CFU:3 neutrophils were added to 400mcl of neutrophils suspended in RPMI-1640 at a concentration of 1 $x10^6$/ml.

For experiments comparing serum opsonic capacity the methodology was modified to resemble opsonophagocytosis assays developed for *S. pneumoniae* [10]. Serum was serially diluted 1:1 with RPMI-1640 during preparation. Neutrophils were resuspended at 2 $x10^6$/ml and 200mcl of this suspension were added to tubes together with 250mcl pre-diluted serum and 50mcl organisms. This modification allowed a maximum serum concentration of 50%, although preliminary experiments indicated maximal internalisation 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 internalisation at concentrations between 1 in 8 and 1 in 32 for non-heat-inactivated serum). Dilutions then proceeded two-fold. To ensure adequate internalisation even with heat-inactivated serum, a higher MOI was used of 3 CFU:1 neutrophil. Each pair-wise comparison of an HIV-1-infected and HIV-1-uninfected serum utilised a single donor's neutrophils. In addition to the percentage of neutrophils internalising organisms at each serum concentration we also calculated a 'break-point', the

concentration at which internalisation fell to less than 50% of that seen at maximum concentration. If 50% of maximum internalisation was not reached then the break-point was defined as two-fold further dilution than the last dilution tested. We also repeated the analysis excluding those samples which failed to reach break point.

After samples were prepared they were incubated for 30 minutes on rocking plates at 37^{o}C and then processed at 0^{o}C as described [9]. The flow cytometric analysis is summarised 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): 10mcl anti-CD4 (control), 0.5mcl anti-CD11a, 10mcl anti-CD16, 10mcl anti-CD35 and 0.5mcl anti-CD88 (all from Becton Dickinson).

Statistical analysis

Two groups were compared using Student's t-test , three or more groups were analysed by one-way ANOVA. Statistical analysis was performed using GraphPad Prism v4.0 or later.

Results

CD35 and CD16 are important for internalisation of M.tuberculosis by neutrophils

Pre-incubation of neutrophils from nine donors with anti-CD35 or anti-CD16 antibodies reduced internalisation of FITC-labelled *M.tuberculosis* versus anti-CD4 controls in the presence of pooled healthy donor serum (Figure 1B). The mean ±SD percentage of neutrophils internalising 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 internalising of 13.3 ±5.4% ($p<0.001$ versus controls; Figure 1B).

Blockade of CD11a or CD88 does not affect internalisation of M.tuberculosis by neutrophils

Pre-incubation of neutrophils from eight donors with anti-CD11a or anti-CD88 antibodies, singly or in combination, did not affect internalisation of FITC-labelled *M.tuberculosis* versus anti-CD4 controls (Figure 1C). The mean ±SD percentage of neutrophils internalising 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 ($p\geq0.18$ for all comparisons). In preliminary experiments there was no effect on phagocytosis even of higher concentrations of these antibodies (anti-CD11a up to 5µL and anti-CD88 up to 10µL).

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 (via CD35) and antibodies (via 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 eight antiretroviral-naïve HIV-1-infected and HIV-1-uninfected donors for use in the phagocytosis assay. Supplementary Table 1 provides donor demographic details.

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

For non-heat-inactivated serum there was no difference in maximal internalisation, but at further dilutions of serum the mean ±SD percentage of neutrophils internalising organisms was significantly reduced using HIV-1-infected patients' serum (at final concentration 1 in 32: 40.9 ±37.0% vs non-HIV-1-serum, 77.0 ±13.7%, p=0.016; at concentration 1 in 64: 31.5 ±37.0% vs 72.3 ±14.0%, p=0.010; at concentration 1 in 128: 24.7 ±30.8% vs 65.1 ±16.9%, p=0.009; Figure 2B). Beyond these concentrations, there was no difference between the two groups (although we were unable to assess all donors at these concentrations).

Correspondingly, the 'break-points' in the HIV-1-infected group were seen at significantly higher concentration (mean ±SD for HIV-infected 1 in 120 ±113 vs HIV-uninfected 1 in 384 ±274, p = 0.048; Figure 2C). There remained a significant difference after excluding 2 samples which did not reach break point: mean ±SD for HIV-infected 1 in 120 ±113 vs 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 especial 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 internalisation.

Here we have first confirmed our own [9] 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 (Complement Receptor (CR)-1) before phagocytosis significantly reduced internalisation. By contrast, the C5a receptor CD88 did not appear to play a significant role and nor does CD11a appear 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 internalisation in participants with evidence of sensitisation to *M.tuberculosis* (positive IGRA result, although this may be confounded by HIV status). We have previously demonstrated modest neutrophil phagocytosis of *M.bovis* BCG with 10% heat-inactivated adult human serum but a complete absence of internalisation with heat-inactivated fetal calf serum, which presumably lacks anti-mycobacterial antibodies [9]. This corresponds with findings by others, for example greater neutrophil internalisation in the presence of heat-inactivated serum versus no serum [7] and increased internalisation 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 ART-naïve HIV-1-infected and HIV-1-uninfected individuals. Opsonophagocytosis for *Candida albicans* and *Streptococcus pneumoniae* is impaired by HIV-1 infection [13][14], and we here extend that conclusion to *M.tuberculosis*. Utilising heat-inactivated serum we observed a reduction in maximal internalisation. For non-heat-inactivated serum, there was an earlier reduction in phagocytosis with serial dilution and earlier 'break-points', suggesting global impairment of *M.tuberculosis* opsonisation. 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 via CD16 to reduce CR3 expression [16]. Indeed, we noted in preliminary experiments that incubation with 50% serum yielded consistently lower internalisation 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 internalisation with heat-inactivated serum. As described, there are potential issues with this approach [9], including 'clumping' of organisms. However, these experiments represented direct comparisons between one HIV-1-infected and one non-HIV-1-infected serum sample with other conditions standardised (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 defence 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 internalisation of bacilli should be considered: CD16 or CD35 would be appropriate targets.

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Figure Legends

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 via high CD66a,c,e-PE expression and finally the percentage of neutrophils positive for FITC-labelled *M.tuberculosis* signal is calculated. B. 4 x 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-labelled *M.tuberculosis*-lux at multiplicity of infection 1 CFU: 3 neutrophils. After 30 minutes incubation, samples were processed at 0^{o}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 internalised organisms (positive for FITC signal) Lines represent means. C. Samples from 8 healthy donors were processed as in (B) utilising antibodies against the receptors indicated. Data expressed as in (B).

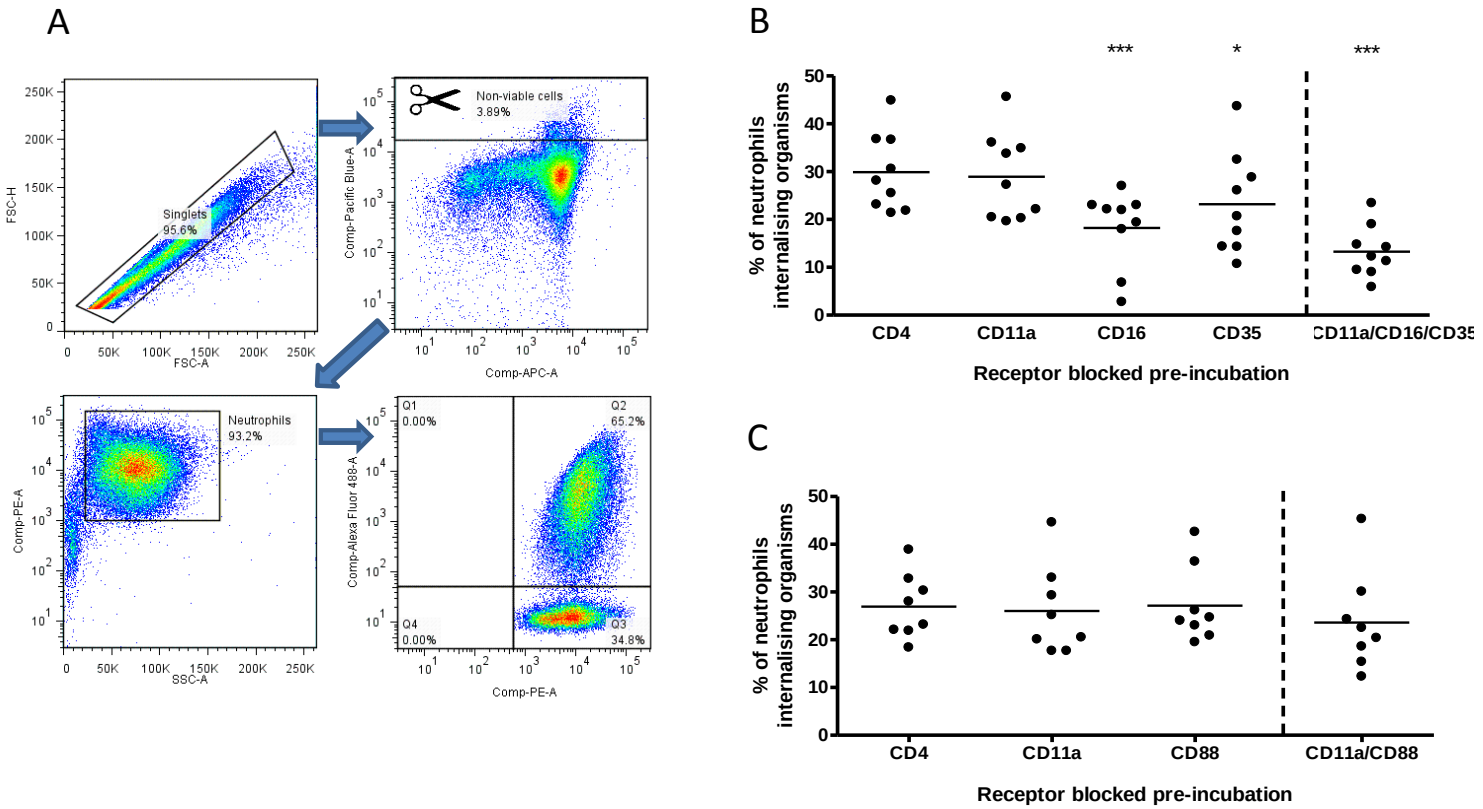
* $p<0.05$, *** $p<0.001$ (Repeated measures one-way ANOVA for individual receptor-blocking conditions, post-hoc Bonferroni correction comparing each neutrophil receptor antibody with anti-CD4 control; paired t-test of 'all' condition versus CD4 controls).

Figure 2. The effect of HIV on the opsonising 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 two-fold with RPMI-1640 before adding 250µL of each concentration to 4 x 10^5 healthy donor neutrophils and FITC-labelled *M.tuberculosis*-lux at multiplicity of infection 3 CFU: 1 neutrophil. The final serum concentration is depicted on the x-axis. After 30 minutes incubation, samples were processed at 0^{o}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 internalised organisms (positive for FITC signal). Each experiment used a single donor's neutrophils and compared one HIV-infected with one HIV-uninfected serum sample. n=8 overall (n=7 for 1/8 dilution) Column heights represent means, 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. 'Break points' were defined from the results of (A) and (B) as the reciprocal of the serum dilution at which internalisation 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)

Figure 1


A
FSC-H
FSC-A
Singlets
95.6%
Non-viable cells
3.89%
Comp-Pacific Blue-A
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Neutrophils
93.2%
Comp-PE-A
SSC-A
Q1
0.00%
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CD4
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CD11a/CD16/CD35
Receptor blocked pre-incubation
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CD4
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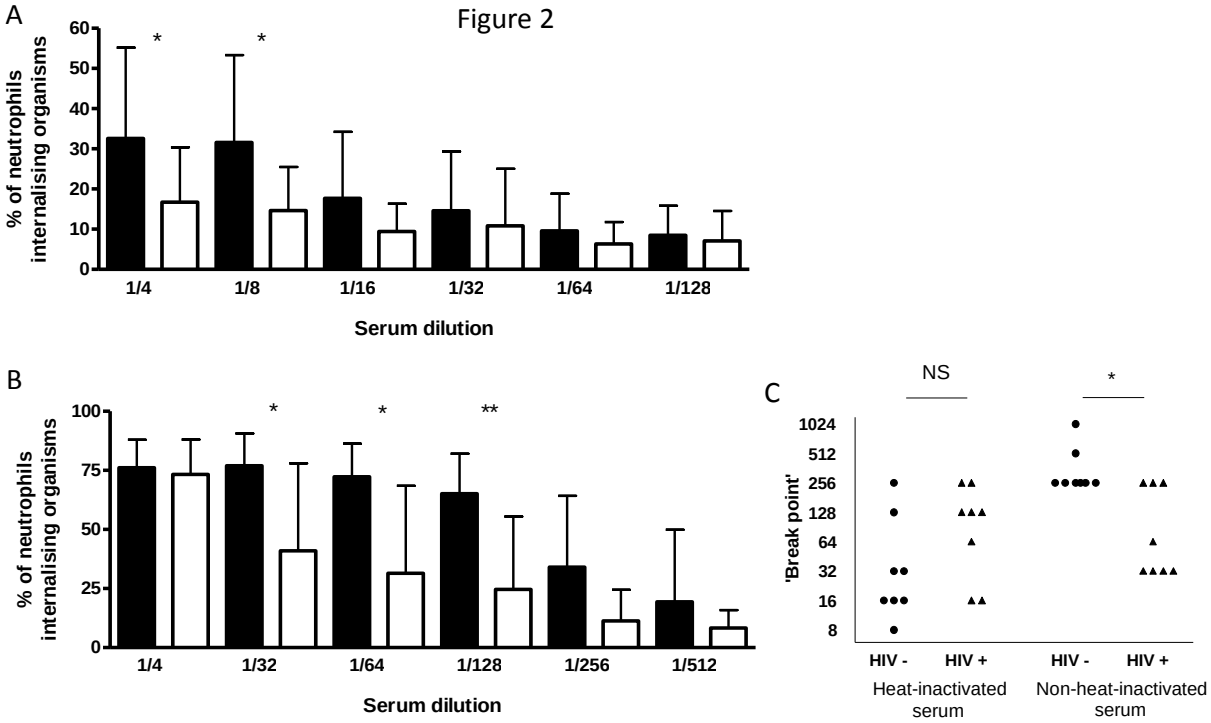
Receptor blocked pre-incubation

Figure 2


A
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'Break point'
1024
512
256
128
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32
16
8
HIV -
HIV +
HIV -
HIV +
Heat-inactivated serum
Non-heat-inactivated serum

Supplementary Information

Supplementary Table 1. Participant characteristics of donors providing HIV-1 infected versus HIV-uninfected serum.

		HIV-infected (n = 8)	HIV-uninfected (n = 8)
Age (years)	Median	34	26
	Range	26 – 70	21 – 36
Sex	Male	2 (25.0%)	2 (25.0%)
	Female	6 (75.0%)	6 (75.0%)
Current Smoking	Yes	1 (12.5%)	1 (12.5%)
	No	7 (87.5%)	7 (87.5%)
Regular Alcohol *	Yes	3 (37.5%)	2 (25.0%)
	No	5 (62.5%)	6 (75.0%)
BCG	Yes	6 (75.0%)	4 (50.0%)
	No	0 (0%)	2 (25.0%)
	Unsure / Unclear	2 (25.0%)	2 (25.0%)
Co-morbidity †	Yes	5 (62.5%)	3 (37.5%)
	No	3 (37.5%)	5 (62.5%)
Co-trimoxazole prophylaxis	Yes	8 (100%)	N/A
	No	0 (0%)	
Vitamin B Co-Strong	Yes	8 (100%)	N/A
	No	0 (0%)	
Other medication in previous 3 months ‡	Yes	4 (50.0%)	2 (25.0%)
	No	4 (50.0%)	6 (75.0%)
Interferon-gamma release assay result	Positive	2 (25.0%)	4 (50.0%)
	Negative	6 (75.0%)	3 (37.5%)
	Unknown	0 (0%)	1 (12.5%)
CD4 count (x10^6/L)	Median	115	N/A
	Range	22 – 180	
Log [HIV viral load (copies/ml)]	Median	5.4	N/A
	Range	3.9 – 6.06	

* Defined as at least once per week

† Identified co-morbidities: Anaemia, oral candidiasis, hypertension, sinusitis, asthma

‡ Identified medications: Ferrous sulphate, folic acid, amitriptyline, antihypertensives (unknown class), nystatin, salbutamol, perindopril

BCG = *Mycobacterium bovis*-BCG; CD4 = Cluster of Differentiation-4; HIV = Human Immunodeficiency Virus; N/A = Not Applicable.