Journal of Immunological Methods 457 (2018) 41–52

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Neutrophils mediate HIV-specific antibody-dependent phagocytosis and ADCC

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ARTICLE INFO

Keywords:
HIV-1
Neutrophils
HL-60
ADCC
Phagocytosis
Antibody

ABSTRACT

There is growing evidence to support the role of Fc-mediated effector functions, such as Antibody-Dependent Cellular cytotoxicity (ADCC) and Antibody-Dependent Phagocytosis (ADP) in the protection and control of HIV. The RV144 trial and other recent HIV vaccine studies have highlighted the importance of ADCC responses in protection against HIV. The role of neutrophils, the most abundant leukocyte in the blood, has not been thoroughly evaluated for Fc-mediated effector functions to HIV. We optimized HIV-specific neutrophil ADCC and Antibody-Dependent Neutrophil Phagocytosis (ADNP) assays using freshly isolated primary human neutrophils from blood. We also developed methods to study ADP using the neutrophil-like HL-60 cell line. We found that neutrophils mediate both HIV-specific ADP and ADCC responses. In vitro, neutrophil-mediated ADCC responses peaked at 4 h, much faster than primary NK cell or monocyte-mediated responses. We detected a wide range of responses in the ADNP, HL-60 mediated ADP and ADCC across a cohort of 41 viremic antiretroviral therapy naïve HIV positive subjects. HL-60 and Neutrophil-mediated ADP and ADCC responses correlated well with each other, suggesting that they measure overlapping functions. The ADNP and HL-60 ADP inversely correlated with HIV viral load, suggesting that these antibody-mediated neutrophil-based assays should prove useful in dissecting HIV-specific immunity.

1. Introduction

HIV-specific antibodies with Fc-mediated functions may play an important role in protection from HIV. Recent human and non-human primate HIV vaccine studies have identified Fc receptor (FcR) mediated responses as potential correlates of protective immunity (Haynes et al., 2012; Bradley et al., 2017; Chung et al., 2015; Barouch et al., 2013, 2015). In non-human primate vaccine studies ADP and ADCC responses have correlated with protection (Bradley et al., 2017; Barouch et al., 2012, 2013). The importance of ADCC and ADP mediating antibodies during HIV infection has also been highlighted by numerous studies correlating ADCC and ADP with decreased disease progression (Lambotte et al., 2009; Baum et al., 1996; Wren et al., 2013; Chung et al., 2011a; Vaine et al., 2010; Ana-Sosa-Batiz et al., 2014; Ackerman et al., 2013). In addition, the moderately efficacious RV144 human Phase III vaccine trial demonstrated the potentially protective capabilities of FcR mediated responses, with ADCC responses correlating with reduced risk of infection in the absence of IgA (Haynes et al., 2012). Multiple different effector cells, including plasmacytoid dendritic cells (pDC) (Tjiam et al., 2015), NK cells (Chung et al., 2011a), monocytes/macrophages (Kramski et al., 2012a), other dendritic cell subsets (Altfeld et al., 2011) and neutrophils (Smalls-Mantey et al., 2013), have the capacity to mediate potent anti-viral Fc-effector responses against HIV. Recent research suggests that polyfunctional Fc-effector responses (i.e. the capacity to engage and recruit multiple different effector cells and functions) may be associated with protection

Abbreviations: HIV-1, human immunodeficiency virus 1; ADP, antibody-dependent phagocytosis; ADNP, antibody dependent neutrophil phagocytosis; ADCC, antibody dependent cellular cytotoxicity; RFADCC, rapid-fluorometric Antibody dependent cellular cytotoxicity; CFSE, carboxyfluorescein succinimidyl ester; FcR, Fc receptor; DMSO, dimethyl sulfoxide; ART, antiretroviral therapy; SHIP, specific hybridisation internalization probe

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https://doi.org/10.1016/j.jim.2018.03.007
Received 19 December 2017; Received in revised form 28 February 2018; Accepted 20 March 2018
Available online 29 March 2018
0022-1759/

and control of HIV infection (Ackerman et al., 2016, Chung et al., 2014). However, the majority of Fc-effector studies in HIV focus upon examining NK cells and/or monocytes responses, while other effector cells such as neutrophils remain understudied.

Neutrophils are the most abundant circulating leukocyte in the blood (30–70%) and can rapidly migrate to sites of infection and can mediate a range of effector responses (Kolaczkowska and Kubes, 2013; Mantovani et al., 2011; Palmer et al., 2006). Although neutrophil functions have long been associated with the killing and control of bacterial and fungal infections, there is growing interest in their role in control of viral infections (Mocsai, 2013; Galani and Andreakos, 2015; Naumenko et al., 2018). In the context of HIV infection, which is predominantly a mucosally acquired infection, it is important to note that neutrophils are abundantly present at mucosal surfaces, especially vaginal tissues and their presence is upregulated during HIV infection (Somsouk et al., 2015; Sips et al., 2016). Neutrophils express FcγRI (induced by cytokines) (Bovolenta et al., 1998), FcγRII and FcγRIII and can mediate a range of antibody-dependent effector functions, including ADP and phagocytosis-independent ADCC responses, however little is known of their importance in HIV infection (Bradley et al., 2017; Ackerman et al., 2016; Sips et al., 2016). Furthermore, while neutrophils have previously been reported to mediate ADCC killing of HIV infected cells, it remains unclear if this was in part mediated by phagocytosis (Baldwin et al., 1989; Jenkins et al., 1993).

NK cells have been extensively studied for ADCC responses (Isitman et al., 2012; Seidel et al., 2013), and mediate target cell lysis through the release of perforin and granzymes (Bryceson et al., 2006). In contrast, the mechanisms that neutrophils utilize to mediate ADCC remains controversial, as they lack perforin and granzyme (Grossman and Ley, 2004; Metkar and Froelich, 2004). Neutrophils release reactive oxygen intermediates following crosslinking of FcR which have been associated with ADCC responses (Horner et al., 2007). However, neutrophils isolated from chronic granulomatous disease patients lack reactive oxygen intermediates but are still able to mediate ADCC responses (Roberts et al., 1993). This indicates that reactive oxygen intermediates, contribute to, but are not the sole mechanism of neutrophil ADCC responses.

The rapid-fluorometric ADCC (RFADCC) has been used extensively to evaluate ADCC responses with monocytes, NK cells and PBMCs (Vaine et al., 2010; Gomez-Roman et al., 2006; Chung et al., 2009, 2011b; Ruiz et al., 2016; Lai et al., 2014). The short half-life of fresh primary neutrophils (6–8 h) means that it would be useful to develop cell-line based neutrophil assays (Summers et al., 2010). The human promyelocytic leukemia HL-60 cell line has the capacity to be differentiated into neutrophil-like CD11b+ cells after culture with dimethyl sulfoxide (DMSO) (Chang et al., 2006; Collins et al., 1978), and can be used to evaluate neutrophil effector functions (Yaseen et al., 2017; Fleck et al., 2005; Kim et al., 2015).

In this study, we optimized and validated HIV-specific neutrophil-mediated RFADCC assays and ADP assays and developed a neutrophil-like cell-line based HL-60 ADP assay. IgG purified from plasma of 41 viremic antiretroviral therapy (ART) naïve HIV positive subjects were readily able to mediate ADNP, HL-60 ADP and neutrophil RFADCC responses. Furthermore, these Fc-mediated neutrophil responses inversely correlated with viral load, suggesting that these optimized assays should prove useful in the evaluation of immune responses, where functional antibodies and neutrophils may play an important role.

2. Material and methods

2.1. Study subjects/plasma samples

Plasma was collected from HIV positive ($n = 41$) subjects, previously described (Wren et al., 2013; Chung et al., 2011a), and HIV negative healthy donors ($n = 13$). All HIV positive subjects were antiretroviral therapy (ART) naive. HIV positive subjects had a median CD4 T cell count of 520 cells/μl (range 296–1156 cells/μl) and a median plasma HIV-1 RNA level of 26,700 copies/ml, (range 399–339,000 copies/ml) to reflect the spectrum of HIV disease states (Table 1). All subjects provided written informed consent and the studies were approved by the relevant institutional ethics committees.

Table 1
Clinical characteristics of study cohort.

	HIV positive cohort
Number of subjects	41
Median CD4 count entry, cells/μl (range)	520 (296–1156)
Median plasma HIV-1 RNA copies/ml (range)	26,700 (399–339,000)

2.2. Primary cell isolation and culture

Neutrophils were isolated by adapting previously published methods (Bowers et al., 2014; Nauseef, 2007). Briefly, fresh heparinized blood was obtained from HIV negative donor leukocytes, separated using Ficoll density centrifugation. The granulocytes were enriched for by using 3% dextran sedimentation for 25 min at room temperature. The remaining cells were washed in and suspended in RPMI 1640 media supplemented with 10% FCS and penicillin (100 U/ml)/Streptomycin/(100 μg/ml) and L-Glutamine (2 nM) (Gibco, 10378-16) (RF10). The enriched cells were then collected and treated with a hypotonoic lysis reagent to remove any remaining red blood cells. The purity of the isolated cells was evaluated by staining with anti-CD16 BV605, CD3 PerCP (Biolegend), CD32 FITC, CD89 APC, CD64 BV510, CD66 BV421, CD14 APC-H7 and CD56 PE (BD biosciences). Monocytes and NK cells were isolated with RosetteSep kits (Stem Cell Technologies) as per manufacturer's instructions.

2.3. HL-60 cells maintenance and differentiation

HL-60 cells (ATCC) were cultured in Iscove's Modified Dulbecco's Medium (Sigma) with 20% heat-inactivated FCS and penicillin (100 U/ml)/Streptomycin/(100 μg/ml) and L-Glutamine (2 nM). To differentiate the HL-60 cell into a neutrophil-like subset, sterile DMSO (Sigma) was added into media at a final concentration of 1.3%, culturing for five days, as previously described (Collins et al., 1978; Martin et al., 1990; Birnie, 1988). The generation of neutrophil-like cells was assessed by staining with CD11b (Chang et al., 2006; Collins et al., 1978), a marker that has previously been identified as essential for neutrophil Fc receptor-mediated cytotoxicity (van Spriel et al., 2001).

2.4. IgG antibody purification

Total IgG was purified using Melon gel resin (Thermo Scientific) following the manufacturer's instructions. Briefly, purification columns (Pierce/Thermo Scientific) were prepared by loading 500 μl of Melon gel resin and washed with Melon gel purification buffer (Thermo Scientific). Plasma samples were diluted 1:5 in purification buffer, added to columns and incubated at room temperature on an orbital rotator for 5 min. The flow-through was collected then placed back into the column and incubate at room temperature for a further 5 min on the orbital rotator. The columns were centrifuged for 1 min at 3000*g* to elute the purified IgG.

The purified antibodies were quantified using an anti-IgG ELISA kit (Mabtech) following the manufacturer's instructions. Briefly, Maxisorb 96 wells plates (Nunc) were coated with the MT145 (2 μg/ml) capture antibody overnight at 4 °C. The plate was washed with PBST (PBS with 0.05% Tween 20) and blocked with 1% BSA/PBST for 2 h. The plate was washed and purified IgG antibodies diluted 1:20000 and 1:50,000 in 1% BSA/PBST were added for 2 h, alongside the IgG standard. The plate was washed and the MT78-ALP secondary added to each well and

incubated for 1 h at room temperature. The plate was washed five times and developed using of *p*-nitrophenyl-phosphate (pNPP) and detected for optical density at 405 nm on a Thermo Fisher Multiskan Ascent plate reader.

2.5. Rapid fluorometric antibody-dependent cellular cytotoxicity (RFADCC)

The RFADCC was performed using similar methods to previously published methods (Kramski et al., 2012b) with different effector cells. Briefly, 1×10^6 CEM.NKr-CCR5 cells in 50 µl of RF10 medium were coated in 3 µg of HIV-1_{BAL} envelope protein gp120 (NIH AIDS reagent program) by incubation at room temperature for 1 h. Uncoated CEM.NKr-CCR5 cells were treated identically but without the addition of the $gp120_{BAL}$. The cell membrane of coated and uncoated CEM.NKr-CCR5 cells were initially stained with 7.5×10^{-7} M of PKH26 (Sigma) diluted in diluent-C for 5 min, stopped with 500 µl of FCS and washed twice with PBS. The cytosols of coated and uncoated CEM.NKr-CCR5 cells were then stained with 1×10^{-6} M CFSE (Sigma) for 3 min, stopped with 500 µl of FCS and washed twice with PBS, then suspended in RF10 media. 2×10^4 PKH26+ CFSE+ coated and uncoated CEM.NKr-CCR5 cells per well were incubated with the purified IgG for 15 min at room temperature to allow for opsonization of target cells. 2×10^5 effector cells (freshly isolated neutrophils, monocytes, NK cells or PBMCs) were then added to the target cells and incubated for a range of times between 0 and 5-5 h at 37 °C with CO_2. Post incubation, the cells were immediately placed on ice and then fixed with a final concentration of 2% formaldehyde and the proportion of PKH26+ cells that had lost intracellular CFSE staining (lysed target cells) was determined using flow cytometry on a BD LSR Fortessa with high-throughput sampler.

2.6. Antibody-dependent neutrophil phagocytosis (ADNP)

HIV-1_{BAL} gp120 proteins were biotinylated using the EZ-Link Sulfo-NHS-LC biotinylation kit (Thermo scientific) using a 50 mmol excess biotin according to manufacturer's instructions. After the reaction, the free biotin was removed by buffer exchange using Amicon 30 kDa centrifugal filters (EMD millipore). The biotinylated $gp120_{BAL}$ was used to coat the binding sites of 1 µm fluorescent NeutrAvidin Fluosphere (Invitrogen) overnight at 4 °C. The excess antigen was removed by washing the beads with 2% BSA/PBS and diluted 1:100 in 2% BSA/PBS. Then 10 µl of the diluted beads were incubated with the purified IgG for 2 h at 37 °C. The purified neutrophils were added to the bead/antibody mix and incubated for a range of times between 0.5 and 5 h at 37 °C with CO_2 to allow for phagocytic uptake. The cells were then fixed with a final concentration of 2% formaldehyde and cells were acquired by flow cytometry on a BD LSR Fortessa with a high-throughput sampler attachment. The data was analyzed using FlowJo version 9.8.5 and the phagocytic score (% bead positive cells × mean fluorescent intensity)/10^4 was calculated as previously described (Darrah et al., 2007) and was used to compare between conditions.

2.7. HL-60 antibody-dependent phagocytosis

The HL-60 ADP was performed using a similar method to the ADNP. $gp120_{BAL}$ coated NeutrAvidin Fluospheres were prepared in the same manner as the ADNP. The diluted beads were then incubated with the purified IgG for 2 h at 37 °C. The differentiated HL-60 were washed and resuspended in fresh media then 1×10^5 HL-60 cells were added to the beads/IgG mix and incubated for 20 h overnight. The HL-60's were then stained with CD11b BV785 (1:100) and fixed with a final concentration of 2% formaldehyde and acquired by flow cytometry on a BD LSR Fortessa.

2.8. HL-60 ADP-SHIP (specific hybridisation internalization probe) assay

To confirm that the fluorescent beads were completely phagocytosed, the HL-60 ADP-SHIP (specific hybridization internalization probe) assay was performed as previously described for THP-1 cells (Ana-Sosa-Batiz et al., 2014). Briefly, 3 µg biotinylated $gp120_{BAL}$ (NIH AIDS Reagent Program) were incubated with 1 µl NeutrAvidin Fluosphere 1 µm beads (Invitrogen) and 1 µl of 150 mM biotin- and Cy5-labelled florescent internalization probe (FIPCy5) (5′ Cy5-TCAGTTCA GGACCCTCGGCT-N3 3′, Integrated DNA Technologies) overnight at 4°C. The gp120-coated beads were washed twice with sterile 2% PBS-BSA and diluted in 100 µl 2% PBS-BSA. Then 10 µl of the diluted beads were incubated with the purified IgG for 2 h at 37 °C. Opsonized beads were incubated with 1×10^5 DMSO-stimulated HL-60 cells in a total volume of 100 µl in fresh media. After incubation for a range of times between 1 and 20 h at 37°C, cells were stained with CD11b BV785. Surface-bound beads were quenched by adding 1 µg/ml^{-1} of the complementary quenching probe (QPC) (5′ -AGCCGAGGGTCCTGAAC TGA-BHQ2- 3′ Integrated DNA Technologies) for 10 min at 4°C and were subsequently washed with PBS and fixed in 2% formaldehyde. Cells were acquired on a Fortessa LSRII (BD Bioscience) and analyzed using Flowjo.

2.9. Confocal imaging

Samples used for microscopy were treated the same way as described for the neutrophil RFADCC and the ADNP assay with the exception that a membrane staining step was performed before fixing. Following the neutrophil RFADCC for 4 h, the cells were stained with CD66b APC, CD89 APC and CD11b APC. While the ADNP samples were stained with CD16 APC and CD32 APC. The samples were washed and fixed with 2% formaldehyde, then loaded on to poly-L-lysine (Sigma) coated slides and cover slips mounted with Ultramount no.4 (Fronine). The Cells were visualized on Zeiss LSM710 laser scanning confocal microscope and images were analyzed using ImageJ.

2.10. Statistical analysis

Statistical analyses were completed using Prism GraphPad version 7.0a (GraphPad Software, San Diego, CA). A Kruskal-Wallis multiple comparisons analysis was used to compare the responses of PBMCs, neutrophils, monocytes and NK cells at each time point. A Mann-Whitney *U* test was used to determine the difference between the HIV positive IgG responses and the HIV negative IgG (healthy donors) for the neutrophil RFADCC, ADNP and the HL-60 ADP. Nonparametric Spearman correlation analyses were used to test for correlations. A *p*-value of < 0.05 was considered to indicate a significant difference.

3. Results

3.1. Purified primary blood neutrophils express a range of Fc receptors

Antibody-mediated neutrophil functions are of interest in HIV immunity since neutrophils can be present at mucosal surfaces in high frequency (Sips et al., 2016) and antibody-mediated functions can contribute to control and protective immunity to HIV (Wren et al., 2013; Chung et al., 2011a; Rerks-Ngarm et al., 2009). To evaluate models of neutrophil-mediated antibody functions, primary neutrophils were isolated from freshly collected heparinized blood by density gradient and dextran sedimentation from normal healthy subjects. Neutrophils were defined as small, highly granular, FSC low, SSC high, CD66b+, CD32+ and were isolated with a mean purity of 99% (Fig. 1A). The Fc Receptor expression on freshly isolated neutrophils was evaluated, with the median MFI for FcαRI (3471), FcγRI (7882), FcγRII (7079) and FcγRIII (34992) determined (Fig. 1B, C). Neutrophils were isolated from 6 different healthy donors had only slightly varying

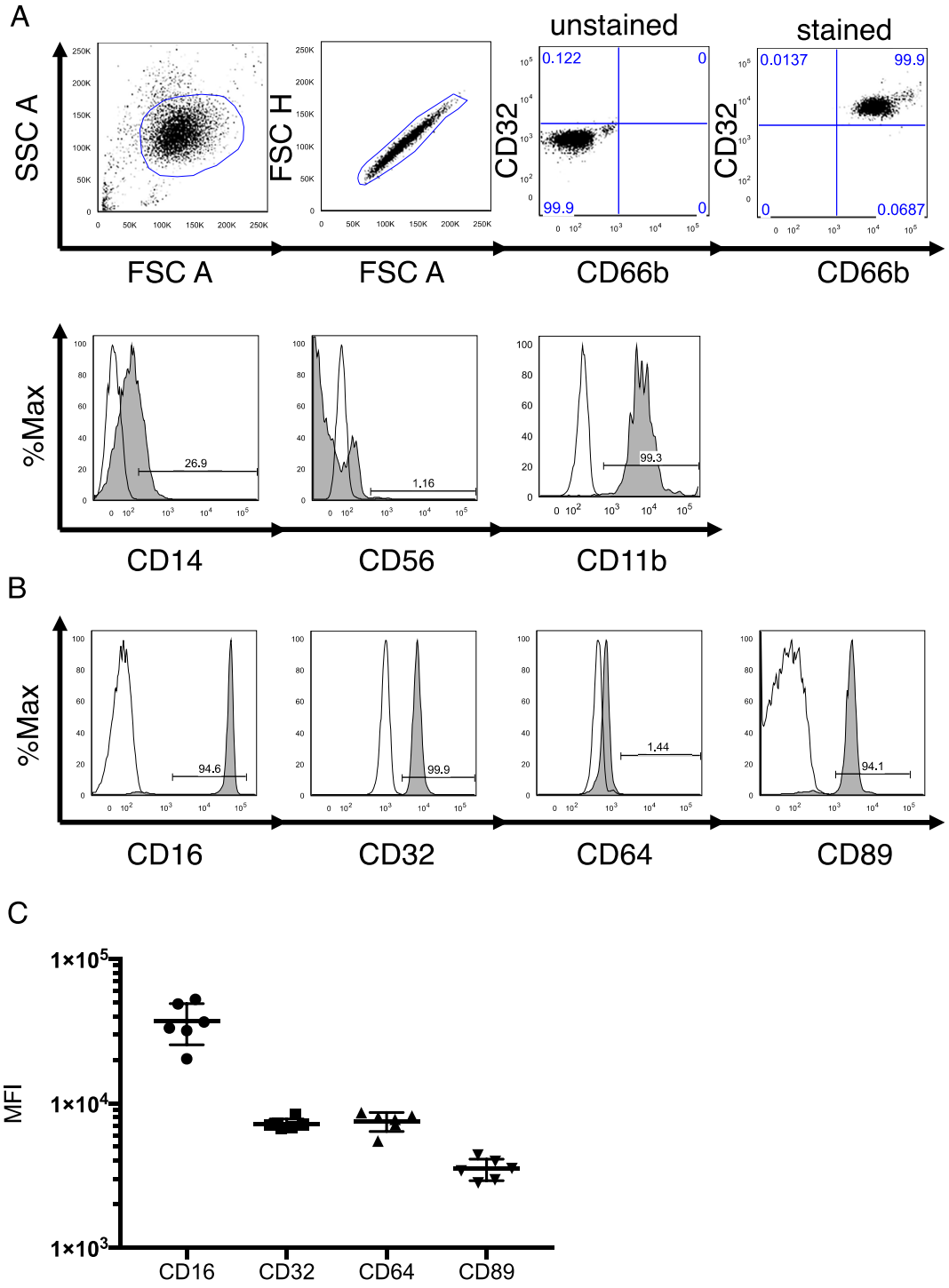


Fig. 1. Purity of isolated neutrophils and Fc receptor expression. A. Representative flow cytometry plots of staining for neutrophil purity for CD66b+ and CD32+. The isolated cells were confirmed to have typical neutrophil phenotypic markers including CD11b+ and CD56- with low levels of CD14. B. Representative flow cytometry plots of isolated neutrophils for the Fc receptors: CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI), CD89 (FcαRI) expression. C. The MFI for Fc receptor surface expression of CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI) and CD89 (FcαRI) on the isolated neutrophils for 6 donors.

levels of Fc receptors present on the surface (Fig. 1C).

3.2. Neutrophil-mediated ADCC as measured by the RFADCC assay

Neutrophils have previously been shown to mediate ADCC in response to antibody opsonized target cells (van der et al., 2002; Peipp et al., 2008) and cancer/tumor cells (Schneider-Merck et al., 2010; Albanesi et al., 2013; Keler et al., 1997), but their ability to mediate ADCC responses against HIV expressing targets has been rarely investigated (Baldwin et al., 1989). To set up a model of neutrophil-dependent HIV-specific ADCC activity, fresh neutrophils were evaluated for their ability to mediate ADCC responses using the rapid fluorometric ADCC (RFADCC) assay (Gomez-Roman et al., 2006). Neutrophils were cultured in the presence of HIV antibodies and HIV-1 envelope protein gp120 $_{BAL}$ coated PKH and CFSE labelled CEM.NKr-CCR5 target cells. Lysis of target cells were defined as PKH+ and CFSE− cells, with a

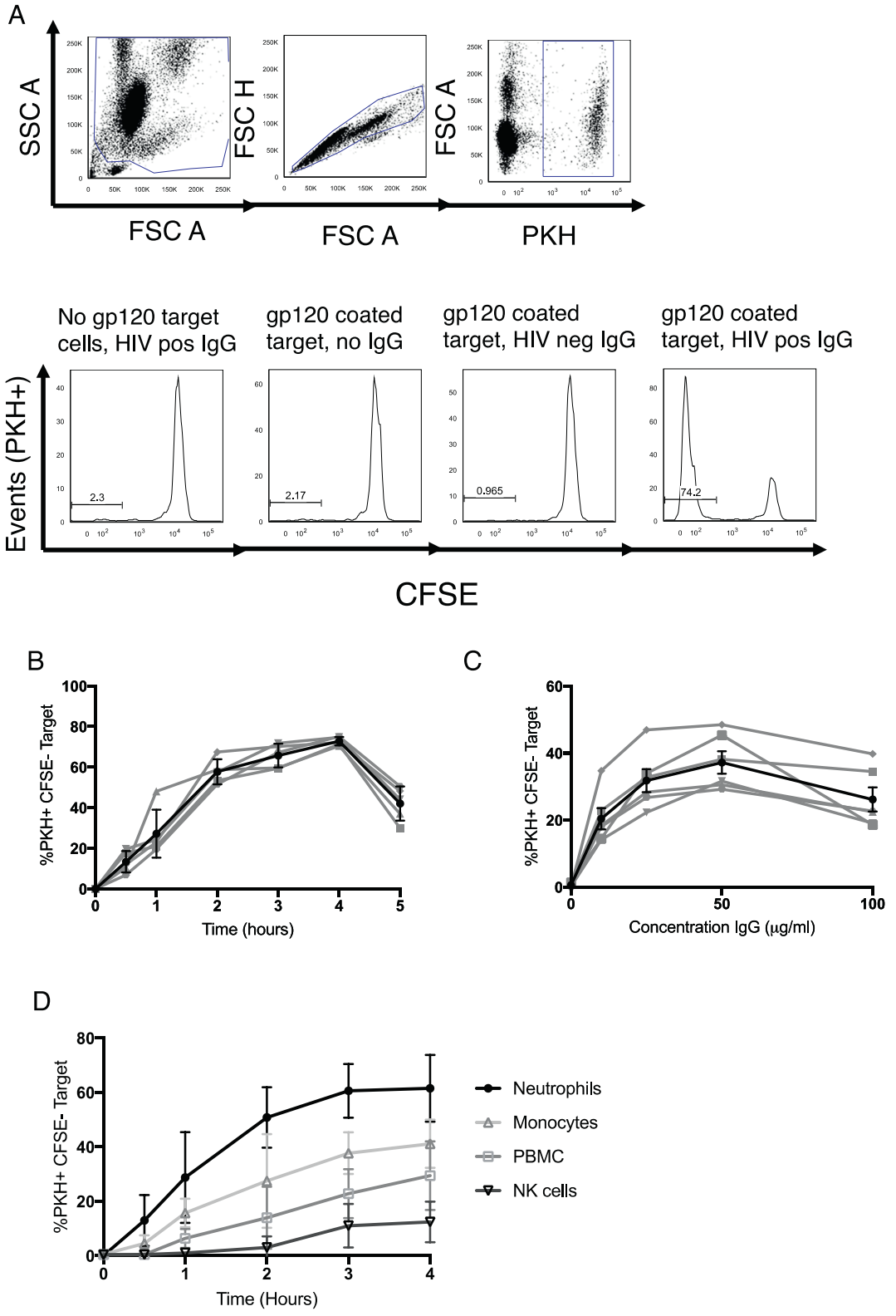


Fig. 2. Neutrophil-mediated RFADCC assay. A. Representative gating strategy for the neutrophil RFADCC. The target cells were stained with PKH membrane dye, CFSE cytoplasmic dye and coated with HIV-1_{BAL} gp120 and incubated with isolated neutrophils. Target cell lysis was identified as cells that were PKH+ and CFSE- with responses only observed for the HIV-1_{BAL} gp120 coated target cells in the presence of HIV positive IgG. B. The %PKH + CFSE- cells at time points 0, 0.5, 1, 2, 3,4 and 5 h utilising 25 µg/ml of a pooled purified IgG from HIV positive donors with 6 neutrophil donors, grey lines each represent different donors, while black line represents the mean response. C. The %PKH + CFSE − cells at different concentrations (100, 50, 25 10 µg/ml) of pooled purified IgG from HIV positive donors with 6 neutrophil donors, grey lines represent different donors, while black line represents the mean response. D. The difference in RFADCC %PKH + CFSE − cell responses with different effectors cells utilising neutrophils, monocytes, NK cells and PBMCs. A Kruskal-Wallis multiple comparisons analysis was performed and showed neutrophils killed more target cells at 0.5 h than NK cells (p = .0008) and PBMCs (p = .0001), at 1 h NK cells ($p \leq .0001$), PBMC (p = .0032), 2 h ($p \leq .0001$), PBMC (p = .0032), 3 h NK cells ($p \leq .0001$), PBMC (p = .0003), 4 h NK cells ($p \leq .0001$), PBMC (p = .0041). The monocytes responded higher than the NK cells at 0.5, 1, 2, 3, 4 h (P = .0018, 0.023, 0.018, 0.023 respectively).

representative gating strategy shown in Fig. 2A. To optimize the assay, we evaluated 5 different healthy neutrophil donors. We found that neutrophils across all donors could readily mediate HIV-specific ADCC and optimal ADCC responses were observed at 4 h, with a median lysis of 72.2% (Fig. 2B).

The neutrophil RFADCC assay was further evaluated to determine the optimal concentration of purified plasma IgG antibody. Purified IgG from 11 different HIV positive donors were evaluated at 4 different concentrations (100, 50, 25 and 10 μg/ml) and an EC50 was determined. A spearman nonparametric correlation (r value) compared the 1/EC50 to the percent lysis for each IgG concentration. For the different concentrations of antibody we found the following results: 100 μg/ml $r = 0.2091$ and $p = .5393$; 50 μg/ml $r = 0.5818$ and $p = .0656$; 25 μg/ml $r = 0.8182$ and $p = .0033$ and 10 μg/ml $r = 0.900$ and $p = .0004$ (Fig. S1). We used 25 μg/ml of IgG in future experiments with the HIV positive cohort as this level had the maximum dynamic range of responses. The neutrophil variability (donors $n = 6$) at different IgG concentrations were evaluated with maximal responses occurring at 50 μg/ml and decreasing at 100 μg/ml showing a prozone effect (Fig. 2C).

A potential advantage of neutrophils as ADCC effectors compared to other immune cells is their ability to rapidly mediate effector functions and thereby rapidly limiting viral spread. Isolated neutrophils were evaluated against other isolated immune cells for their potency in mediating lysis of the target cells over 4 h in the RFADCC assay (Fig. 2D). We found in vitro that neutrophils exhibited higher levels of ADCC at all time points compared to NK cells ($p \leq .001$) and PBMC ($p \leq .005$), while the monocytes responded higher than the NK cells at all time points ($p \leq .05$). Neutrophils also induced higher activity than monocytes, however the responses were not significant. This demonstrates the rapid ability of neutrophils to kill HIV-1 gp120 coated target cells.

3.3. Multiple mechanisms of target cell cytotoxicity are measured by the RFADCC assay

The RFADCC assay has been used widely to evaluate HIV-specific lysis of target cells. Interestingly, Kramski et al. showed that the RFADCC also measures phagocytosis of the labelled lysed target cells by monocytes (Kramski et al., 2012b). We hypothesized such a phenomenon may also occur with neutrophils. The neutrophil-mediated RFADCC assay was therefore adapted to include the CD66b granulocyte marker surface staining to determine if the target cell lysis was mediated by ADCC and/or phagocytosis of the target cells using gating strategy in Fig. 3A. The neutrophil-mediated RFADCC at early time-points of 0.5 h observed high levels of PKH+ CFSE+ CD66b+ populations (47.4%), suggesting the potential early phagocytosis of un-lysed PKH+ CFSE+ target cells by the CD66b+ neutrophils. While at the same time-point low levels of PKH+ CFSE− CD66b+ were observed (10.1%) indicating the rapid lysis of phagocytosed target cells or potentially uptake of lysed target cell membranes. This PKH+ CFSE− CD66b+ population rapidly increased to 48.8% at the 1 hour time point, coinciding with the decrease of PKH+ CFSE+ CD66b+ populations (3.74%) (Fig. 3B). Collectively this early association of the neutrophil and target cell (CD66b+ PKH+ CFSE+) changing to a neutrophil and lysed target cell (CD66b+ PKH+ CFSE−) may indicate that the target cell has been phagocytosed.

To further interrogate the mechanism behind the PKH+ CFSE− target cell lysis, confocal microscopy imaging was used to evaluate the neutrophil-mediated RFADCC assay. Fig. 3C presents PKH+ membrane (red) of target cells internalized in the neutrophil (blue) in the presence (Fig. 3C ii) and absence (Fig. 3C iii) of the CFSE (green) showing phagocytosis of a lysed target cell. There is also evidence that traditional ADCC responses are present, with PKH+CFSE− target cells in close proximity to neutrophils (Fig. 3Ci). In addition, different levels of PKH+ membrane were observed within neutrophils which may indicate the neutrophils are phagocytosing PKH+ membranes of dead cells killed by traditional ADCC responses (Fig. 3Cvi).

3.4. HIV-specific ADNP assay

The ADNP has been previously published (Ackerman et al., 2016) but has not been directly compared to neutrophil RFADCC assays. We employed a modification of the previously reported gp120-coated bead-based phagocytosis assays to study ADNP by flow cytometry using the gating strategy shown in Fig. 4A. ADNP was evaluated using a pool of HIV-specific polyclonal antibodies (HIVIG) at different concentrations measuring phagocytosis with different neutrophil donors ($n = 6$) (Fig. 4B). There was little difference between the 50 and 100 μg/ml concentrations over the course of 5 h. However, when ADNP variability (donor $n = 6$) testing for different IgG concentrations were evaluated at 4 h incubation, similar to the RFADCC assay we saw a plateauing of responses at concentrations of 50 μg/ml or higher (Fig. 4C). To determine that the beads were phagocytosed and not associated with the surface cell membrane, bead internalization was confirmed by confocal microscopy. Fig. 4D illustrates bead (green) surround by membrane (blue) showing the beads are phagocytosed. Z-stack movie confirmed complete phagocytosis (Fig. S2). There was no uptake of the beads observed in the absence of HIV positive IgG.

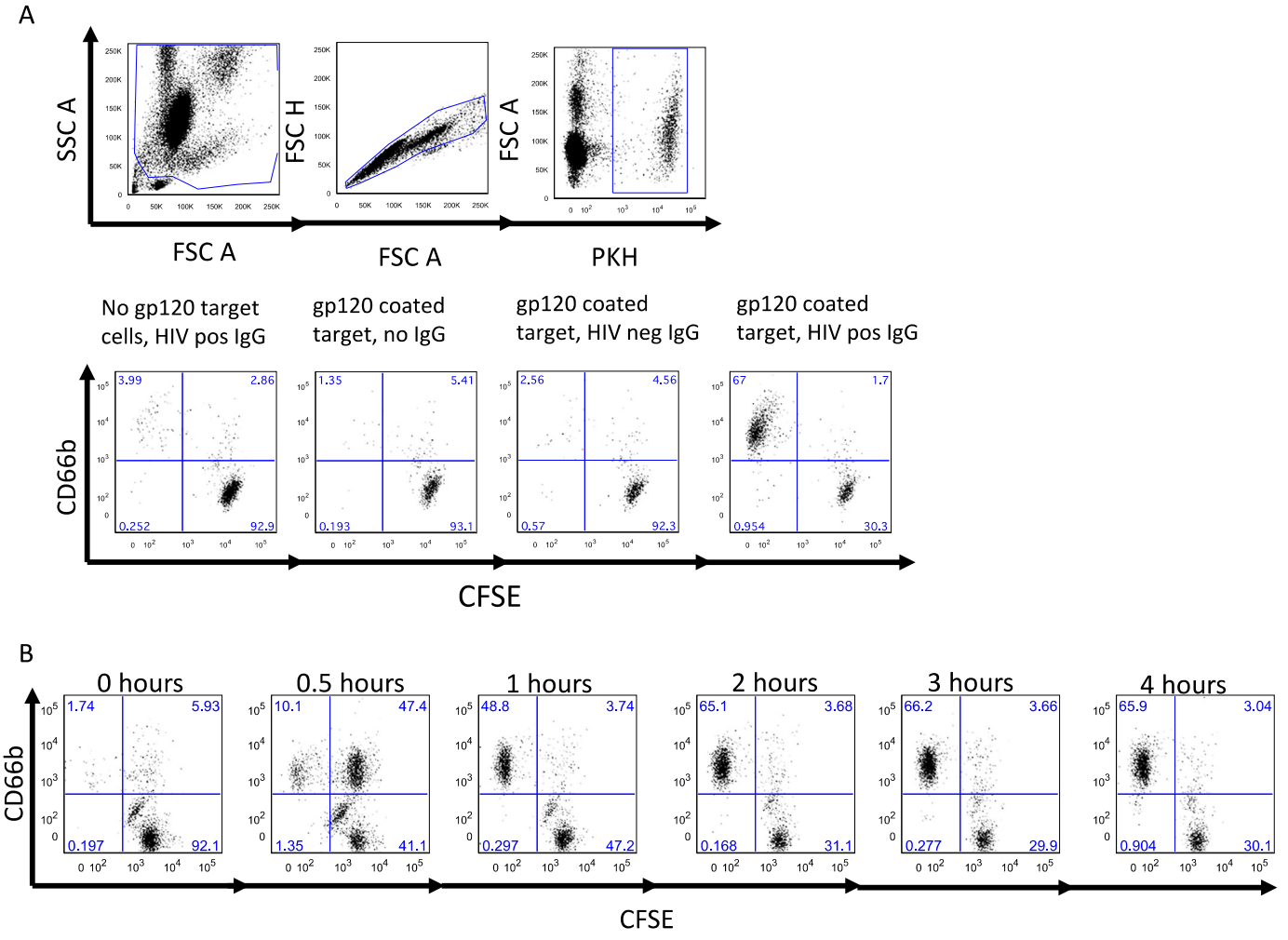
3.5. HIV-specific HL-60 ADP assay

Neutrophils have a short half-life upon isolation, thus requiring constant fresh leukocyte sources for studies. In contrast, the HL-60 neutrophil-like cell line can be continuously grown in culture and differentiated with DMSO when required. We developed a HL-60 ADP assay as a proxy for the evaluation of antibody-dependent primary neutrophil phagocytosis. The HL-60 cells were differentiated with 1.3% DMSO, confirmed by the upregulation of CD11b, a marker that has previously been identified as essential for neutrophil Fc receptor-mediated cytotoxicity and is commonly used as a marker for HL-60 neutrophil differentiation (Birnie, 1988; van Spriel et al., 2001). Furthermore, we confirmed the upregulation of CD14, CD16, CD32, CD89 surface marker expression compared to HL-60 cells prior to differentiation (Fig. 5A). Prior to differentiation, HL-60 cells expressed CD89 (MFI 2178) but had low/no expression of CD11b, CD14, CD16, CD32 and CD64. Following 5 days of differentiation with DMSO, they CD11b (MFI 753) CD89 (MFI 2914), CD14 (MFI 392), CD16 (MFI 641), CD32 (MFI 2204) but with no expression of CD64.

The HL-60 ADP assay gating strategy relies on gating for CD11b positive cells (differentiated HL-60 cells) and then gating on the fluorescent beads (Fig. 5B), while the CD11b negative cells (undifferentiated HL-60 cells) show only low levels of phagocytosed beads. To determine the optimal incubation time of the HL-60 ADP assay, a time-course was completed and showed that maximal responses were reached at 20 h (Fig. 5C). To validate that the beads were internalized, the HL-60 ADP was modified to include a hybridization internalization probe (SHIP) present on the bead (Ana-Sosa-Batiz et al., 2014). Increasing levels of phagocytosis (7.56% at 1 h and 36% at 3 h) were observed over time with 98.6% internalization of the beads observed following a 12 hour period (Fig. 5D).

3.6. HIV positive IgG cohort ADNP, HL-60 ADP and neutrophil-mediated RFADCC

Having established three separate neutrophil-mediated HIV-specific antibody assays, we asked how these assays performed across a cohort of 41 HIV positive subjects who were viremic and not on ART, compared to 13 healthy donors. All three assays clearly discriminated between HIV positive and HIV negative subjects (Fig. 6A). RFADCC results were analyzed, gating on both total PKH+ CFSE− cells and CD66b+ PKH+ CFSE− cells (a measure of Neutrophil uptake of target cells and



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target cell membranes). There were only small marginal differences between the responses with these 2 gating strategies (Fig. 6A).

The ADNP and HL-60 ADP both had a large dynamic range of responses, however, the HL-60 ADP had somewhat greater non-specific uptake of the gp120-coated beads using HIV negative IgG. None-the-less, the ADNP and HL-60 ADP assay exhibited a strong positive

Fig. 3. Neutrophil- mediated RFADCC is a measure of both extracellular cytotoxicity and phagocytosis. A. Alternative gating strategy for the neutrophil RFADCC. Gating on PKH+ CD66b+ and CFSE− shows that CD66b+ neutrophils acquire target cell PKH and this occurs only in the presence of HIV-1_{BAL} gp120 coated target cells and HIV positive IgG. B. Gating on PKH+ cells and then CD66b and CFSE over 0, 0.5, 1, 2, 3 and 4 h utilising 25 μg/ml of a pooled purified IgG from HIV positive donors. C. Confocal microscopy imaging of the neutrophil RFADCC with the neutrophils stained with CD66b APC, CD89 APC and CD11b APC (PKH: red, CFSE: green, Neutrophil markers: blue). Inset panels show i) Dead target cell (PKH+ CFSE−) next to neutrophils, ii) live target cell inside neutrophil, iii) Dead target cell inside of neutrophil, iv) Neutrophil alone, v) CEM.NKr-CCR5 (target) cell alone, vi) small amount of target cell membrane inside of neutrophil. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

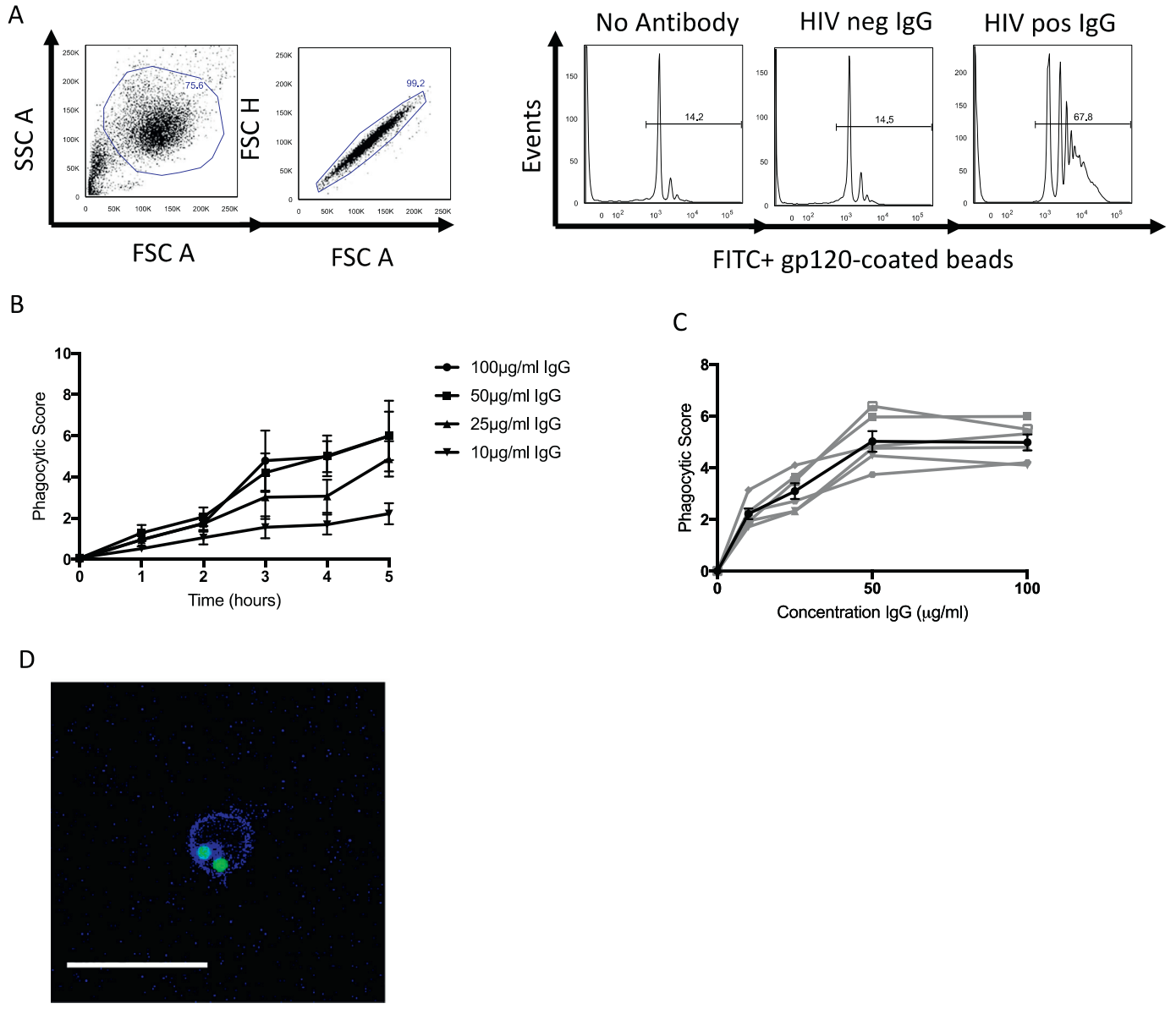

Fig. 4. ADNP responses to HIV positive IgG. A. Representative gating strategy for the ADNP assay. The panels 3 and 4 show minimum uptake of the FITC+ beads in the absence of antibody and in the presence of HIV negative IgG. Panel 5 shows that beads coated with HIV-1_{BAL} gp120 in the presence HIV positive IgG can be taken up by neutrophils. Phagocytic score = %beads positive cells × mean fluorescence intensity/10^4. B. ADNP at time points 0, 0.5, 1, 2, 3, 4 and 5 h utilising different concentrations (100, 50, 25 10 μg/ml) of pooled purified IgG from HIV positive donors with 6 neutrophil donors. C. ADNP responses at 4 h using different concentrations (100, 50, 25 10 μg/ml) of pooled purified IgG from HIV positive donors with 6 neutrophil donors; grey lines represent each donor and black line is mean. D. Confocal microscopy showing the internationalisation of 2 FITC+ beads following a 4 h incubation (neutrophils stained with CD16 APC and CD32 APC), scale bar = 20 μm.

correlation ($r = 0.7718$, $p \leq .0001$, Fig. 6B, right panel). Interestingly, we observed that the neutrophil RFADCC with CD66b gating also showed a significant correlation with both the ADNP ($r = 0.5692$, $p \leq .0001$) and the HL-60 ADP ($r = 0.4524$, $p = .0030$) (Fig. 6B). In addition, the neutrophil RFADCC without CD66b gating also showed a significant correlation with both the ADNP ($r = 0.5655$ $p \leq .0001$) and the HL-60 ADP ($r = 0.4551$, $p = .0028$) (Fig. S3). The same 5 (out of 41) HIV positive donors responded very weakly in all 3 assays.

Since all 41 subjects were ART naive and had a range of HIV viral levels, we were able to evaluated the neutrophil RFADCC, ADNP and HL-60 ADP assays for clinical relevance. We found that there was a significant negative correlation with vial load and ADNP ($r = -0.32$,

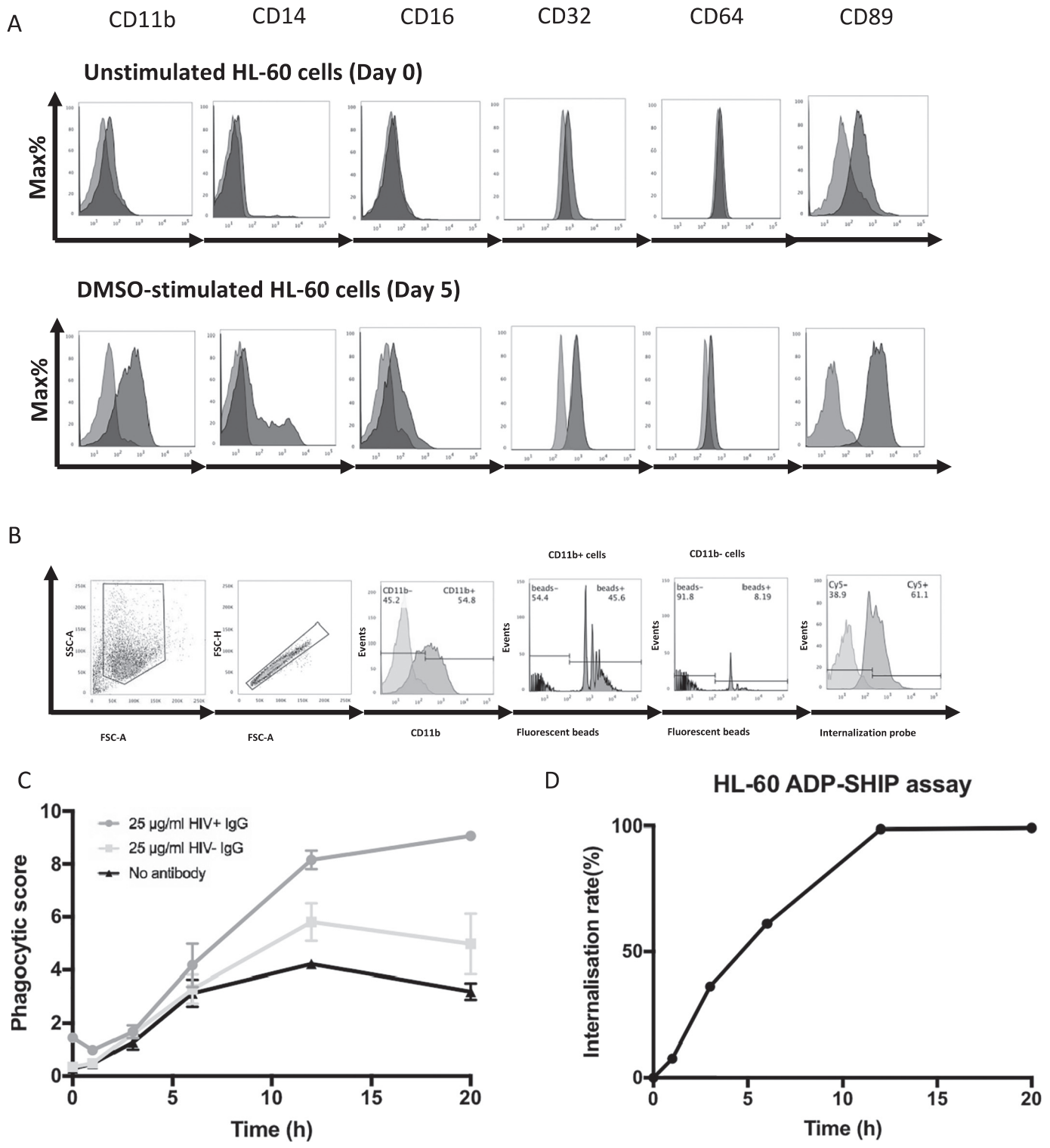

Fig. 5. Differentiated CD11b+ HL-60 cell as a model for ADNP. A. Representative flow cytometry plot for cell markers and Fc receptors profiles in HL-60 cells. D0 were unstimulated naïve HL-60 cells, and D5 were cells stimulated by 1.3% DMSO for five days. Lighter grey populations were unstained control and darker grey populations were stained cells. B. Gating strategy for HL-60 ADP assay and HL-60 ADP-SHIP assay. The first 3 panels show gating strategy on CD11b+ cells. The fourth panel shows the bead (FITC+) phagocytosis activity of CD11b positive HL-60s when cultured with HIV positive IgG and gp120 coated fluorescent beads. The fifth panel shows the bead phagocytosis activity of CD11b negative HL-60s. The sixth panel shows gating on FITC+ cells, identifying internalized beads by gating for Cy5+ internalization probes. FITC+/Cy5+ events were identified as internalized beads and FITC+/Cy5- events were identified as surface-associated beads. C. Time-course plot of HL-60 ADP assay. The phagocytic activity was measured using HIV positive IgG and IgG purified from HIV negative subjects. Phagocytic score = %beads positive cells × mean fluorescence intensity/10^4. The error bars represent mean ± s.e.m of three replicates. D. Time-course plot of beads internalization rate in HL-60 ADP-SHIP assay. Internalization rate% = FITC+Cy5+ cells/ FITC+ cells ∗ 100%.

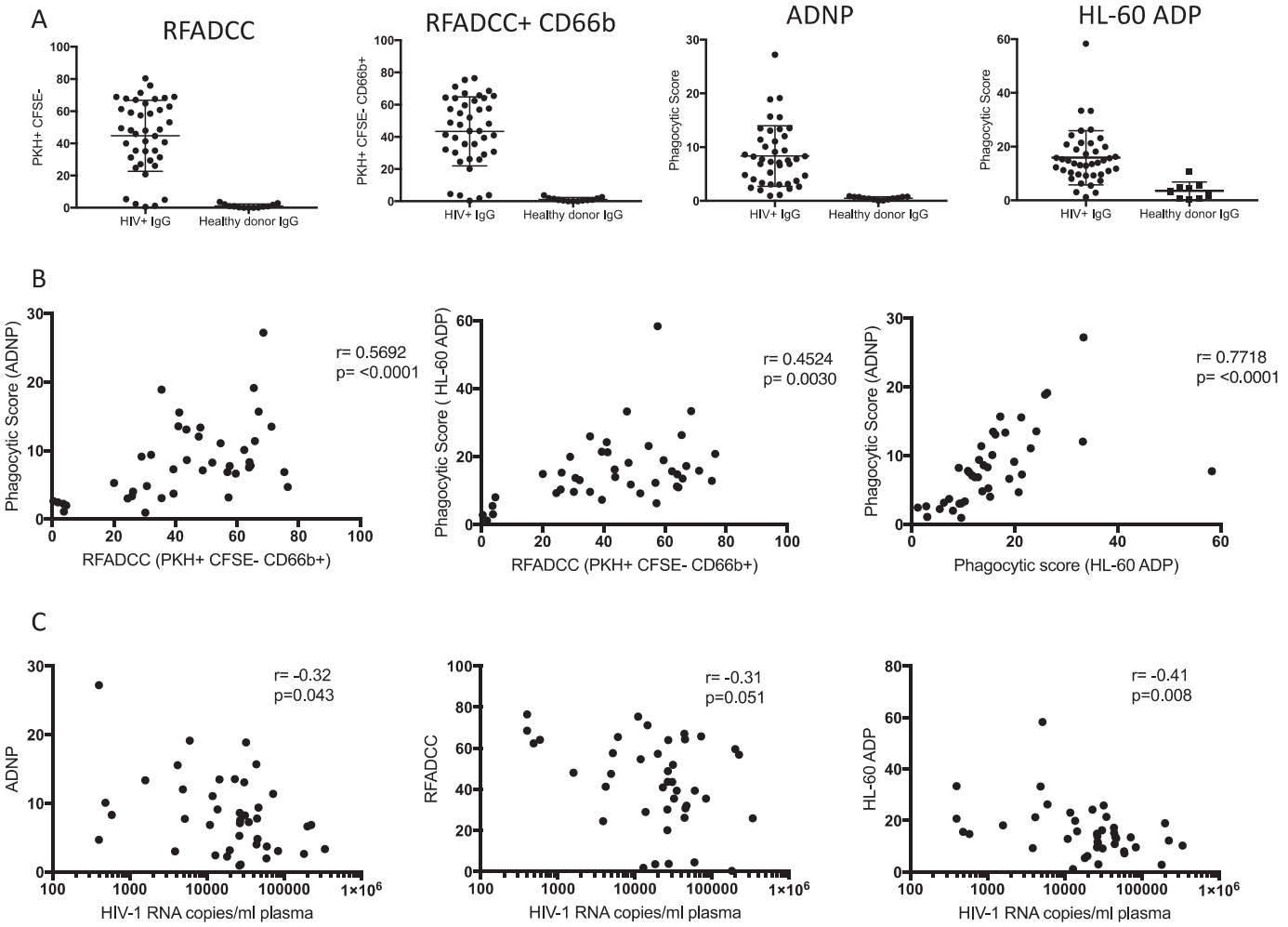

Fig. 6. Neutrophil Fc-Effector responses of HIV positive cohort. A. 41 HIV positive plasma samples and 13 HIV negative plasma samples were purified for IgG and were then assess for responses in the neutrophil RFADCC, neutrophil RFADCC with CD66b gating, ADNP and the HL-60 ADP. B. The HIV positive IgG cohort responses were correlated (spearman) between the 3 assays showing strong significant correlations between the assays. C. The HIV positive IgG cohort responses were correlated (spearman) with viral load (HIV-1 RNA copies/ml plasma) and showed significant negative correlations with the ADNP and HL-ADP and approaching significance with the neutrophil RFADCC with the CD66b gating.

$p = .043$) and the HL-60 ADP ($r = -0.41$, $p = .008$), with the RFADCC approaching significance ($r = -0.31$, $p = .051$) (Fig. 6C). This may indicate that neutrophils play a role in controlling HIV viral load in some patients.

4. Discussion

There is a growing interest in the role of Fc-mediated effector functions, such as ADCC and ADP in the protection and control of HIV. However, there has been limited investigation into the role of neutrophil-mediated Fc-responses in HIV infections. We show that neutrophils are efficient Fc-effector cells and they respond faster than monocytes or NK cells when specifically measured by the RFADCC assay. These differences may be due to the different effector mechanism involved, as neutrophils express a range of FcγRs and can mediate both phagocytosis and ADCC responses, whereas NK cells only express FcγRIIIa and mediate only ADCC responses (Sips et al., 2016; Selvaraj et al., 1988). Since the neutrophil RFADCC is measuring in part phagocytosis responses, it is unsurprising that these responses correlate with the primary neutrophil ADNP assay and neutrophil-like cell line HL-60 ADP assay. The HL-60 neutrophil cell line assay correlated strongly with the primary neutrophil ADNP assay and provides a more tractable model for primary neutrophils in the ADP assays. Importantly, the neutrophil RFADCC, ADNP and HL-60-ADP assays inversely correlated with the viral load of the HIV positive IgG donors at the time of plasma collections, suggesting the possibility that these assays are of biological relevance.

The RFADCC assay has been widely used to assess FcγR mediated responses of PBMCs (Vaine et al., 2010; Gomez-Roman et al., 2006; Chung et al., 2009). While NK cells have been shown to mediate ADCC responses, monocyte responses have largely been attributed to trogocytosis and phagocytosis of the target cells (Kramski et al., 2012b). Our data indicates that the neutrophil RFADCC assay more represents phagocytosis of whole target cells, phagocytosis of killed target cell debris, or trogocytosis. When comparing HIV positive versus negative sample responses, there is an increase in the population of the PKH+ CFSE+ CD66b+ cells (ie neutrophils internalizing live target cells) at early time points (0.5 h) that are higher on the FSC and SSC, which may indicate the target cells have been phagocytosed (Fig. S4). While at the same early time point, there is also a small population of PKH+ CFSE− CD66b+ cells (i.e. neutrophils that have internalized target cell membrane only) which have smaller FSC vs SSC profiles, similar to HIV negative samples, suggesting that the neutrophils have ingested smaller fragments of dead target cells killed by more traditional extracellular ADCC mechanisms. We also observe more PKH+ cells when testing HIV positive IgG samples compared to HIV negative IgG samples,

despite the same number of target cells being added to each well (Fig. S5), which may be caused by neutrophils mediating traditional extracellular ADCC target cell lysis prior to the neutrophil phagocytosing fragments of dead target cells. Alternatively, multiple neutrophils may interact with the same target cell and compete to phagocytose resulting in the sharing of target cell membrane between effector cells.

The neutrophil RFADCC, HL-60-ADP and ADNP responses inversely correlated with the viral load of the patients. ADNP responses have recently been shown not to differ between elite controllers, viremic controllers or patients on antiretroviral therapy (Ackerman et al., 2016). It will be of interest to evaluate the differences in clinical populations in the antibody-mediated neutrophil assays in future studies. In addition, neutrophil phagocytic activity is progressively impaired during HIV infection despite antiretroviral therapy (Tsachouridou et al., 2017). In future studies it will be of interest to investigate neutrophils isolated from different clinical populations of HIV positive donors for their ability to mediated antibody-dependent functions, although these studies are technically demanding due to the need for fresh neutrophils. Our and other studies (Bradley et al., 2017; Smalls-Mantey et al., 2013; Ackerman et al., 2016) have utilized circulating neutrophils isolated from blood, however, it is known that neutrophils residing in tissues and mucosal surfaces can have altered FcγR expression profiles, which could potentially alter the Fc-effector potentials of neutrophils at different sites (Sips et al., 2016) and should be taken into consideration when evaluating FcγR responses. While this study focused exclusively on exploring neutrophil mediated Fc-effector responses, multiple other FcγR bearing innate immune effector cells including pDC, NK cells and monocytes/macrophages may also participate in antiviral control and their respective contributions also deserve further evaluation.

The strong correlation between the ADP and HL-60 ADP assay suggest that HL-60 cells can represent a useful tool in the evaluation of other neutrophil effector functions in the future. Due to the difficulty of working with primary isolated neutrophils that have short half lives and require fresh blood for assays, as neutrophils cannot be reliably cryopreserved without severely effecting functionality (Boonlayangoor et al., 1980), usage of neutrophil-differentiated HL-60 cell lines may allow for easier, more robust, high throughput evaluation of Fc-mediated effector responses. However, HL-60 have several differences compared to primary neutrophils including differences in gene expression and the lack of several neutrophil proteins including the CD66b surface marker (Ozeki and Shively, 2008). In addition, HL-60 neutrophils lack the development of the secondary and tertiary granules which are hall marks of neutrophils, required for some effector functions upon cell activation (Gaines et al., 2005). HL-60 neutrophils have been shown to mediate lower levels of antimicrobial activity and lower reactive oxygen production compared to primary blood derived neutrophils (Yaseen et al., 2017; Watson et al., 1997). These differences should be taken into consideration when utilising HL-60 cells to evaluate alternative Fc-effector functions.

In summary, the neutrophil RFADCC, ADNP or HL-60 ADP were validated, evaluated and compared for neutrophil effector functions. As neutrophil Fc-mediated responses were associated with reduced viral loads in HIV ART naïve subjects, this suggests that neutrophil-mediated effector responses should be investigated in future HIV vaccine trials for their potential to control viremia, especially due to their rapid response compared to other FcγR innate immune cells and their abundance at key sites of HIV transmission. The assays described should help form the foundation for future neutrophil Fc-effector studies in HIV infection and vaccination.

Acknowledgments

We thank Dr. Ivan Stratov for providing HIV positive plasma samples. We would also like to thank Ansari Shaik for assisting with the cohort and clinical information. We acknowledge the NIH AIDS reagents program for supplying the gp120 $_{BAL}$ (cat# 4961), CEM.NKr-CCR5 cells (cat# 4376) and HIV-IG (cat# 3957). We also acknowledge the facilities of the biological optical microscopy platform (BOMP) of the University of Melbourne.

Funding

This work was supported by the Australia National Health & Medical Research Center (APP1125164) and the American Foundation for AIDS Research (amfAR) Mathilde Krim Fellowship (109499-61-RKVA).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.03.007.

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