



## Research paper

# Role of monocytes in mediating HIV-specific antibody-dependent cellular cytotoxicity

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## ABSTRACT

Antibodies (Abs) that mediate antibody-dependent cellular cytotoxicity (ADCC) activity against HIV-1 are of major interest. A widely used method to measure ADCC Abs is the rapid and fluorometric antibody-dependent cellular cytotoxicity (RFADCC) assay. Antibody-dependent killing of a labelled target cell line by PBMC is assessed by loss of intracellular CFSE but retention of membrane dye PKH26 (CFSE-PKH26<sup>+</sup>). Cells of this phenotype are assumed to be derived from CFSE + PKH26 + target cells killed by NK cells.

We assessed the effector cells that mediate ADCC in this assay. Backgating analysis and phenotyping of CFSE-PKH26<sup>+</sup> revealed that the RFADCC assay's readout mainly represents CD3-CD14<sup>+</sup> monocytes taking up the PKH26 dye. This was confirmed for 53 HIV + plasma-purified IgG samples when co-cultured with PBMC from three separate healthy donors. Emergence of the CFSE-PKH26<sup>+</sup> monocyte population was observed upon co-culture of targets with purified monocytes but not with purified NK cells. Image flow cytometry and microscopy showed a monocyte-specific interaction with target cells without typical morphological changes associated with phagocytosis, suggesting a monocyte-mediated ADCC process.

We conclude that the RFADCC assay primarily reflects Ab-mediated monocyte function. Further studies on the immunological importance of HIV-specific monocyte-mediated ADCC are warranted.

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## 1. Introduction

Virus-specific binding but non-neutralizing antibodies (Abs), in particular Abs that mediate antibody-dependent

cellular cytotoxicity (ADCC) activity, have been of major interest in human immunodeficiency virus (HIV) research. Considerable evidence supports a role for ADCC activity in the control of HIV infection with a beneficial impact on disease progression (Ahmad et al., 2001; Huber and Trkola, 2007; Alter and Altfeld, 2009; Chung et al., 2011; Johansson et al., 2011). In the context of vaccination, ADCC-Abs correlate positively with protection in animal models of HIV infection (Gómez-Román et al., 2005; Hidajat et al., 2009; Xiao et al., 2010). Passive transfer experiments in macaques support a role for ADCC in assisting in the control of chimeric SIV-HIV infection (Hezareh et al., 2001; Hessel et al., 2007). The recent partially successful

*Abbreviations:* HIV-1, human immunodeficiency virus type 1; ADCC, antibody-dependent cellular cytotoxicity; RFADCC assay, rapid fluorometric antibody-dependent cellular cytotoxicity assay; CFSE, Carboxyfluorescein succinimidyl ester.

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RV144 clinical HIV vaccination trial (Reerks-Ngarm et al., 2009) also suggested a potential role of non-neutralizing and ADCC Abs in protection (Haynes et al., 2012). Given the potential protective role for non-neutralizing antibodies robust assays are needed to measure HIV-specific ADCC responses.

ADCC responses to HIV-1 were first assayed using the <sup>51</sup>Chromium release assay with envelope (Env) protein coated target cell lines (Baum et al., 1996; Cox, 1999; Cox et al., 1999; Battle-Miller et al., 2002; Nag et al., 2004; Yamada et al., 2004). More recently, several non-radioactive assays measuring ADCC responses have been developed (Sheehy et al., 2001; Pollara et al., 2011). The rapid and fluorometric antibody-dependent cellular cytotoxicity (RFADCC) assay developed by Robert-Guroff and colleagues (Gómez-Román et al., 2006) has been widely used in the context of HIV and SIV vaccine and immunology research (Gómez-Román et al., 2005; Chung et al., 2009; Vaine et al., 2010; Xiao et al., 2010; Patterson et al., 2011). The target cells used in the RFADCC assay are CEM.NKr-CCR5 cells, a CD4+ T cell line resistant to NK cell killing mediated by natural cytotoxicity receptors, that are coated with HIV-1 or SIV Env protein. To assess anti-HIV ADCC activity mediated by HIV-specific IgG present in HIV+ patient sera the Env-coated CEM.NKr-CCR5 are double labelled with the cell membrane dye PKH26 and the cytoplasmic dye CFSE and co-cultured with PBMC from a healthy, HIV-uninfected subject in the presence of defined amounts of test serum. The killing by PBMC is defined by the loss of CFSE but the retention of PKH26 resulting in the emergence of a "killed" PKH26+CFSE- population within the PKH26+ gate.

While using the RFADCC assay, we noted that the population of cells that has previously been accepted as "killed" target cells (PKH26+CFSE-), based on their two-way CFSE-PKH26+ dot plot signature, appeared to emerge from the unlabelled PBMC population, rather than from the PKH26+CFSE+ CEM.NKr-CCR5 target cells. We investigated the PKH26+CFSE- population further and found that antibody-dependent monocyte uptake of PKH26-stained target cell fragments is a more likely explanation of the biological events measured in this assay. An analysis of samples from 53 HIV+ subjects confirmed that gating on PKH26+ monocytes was a more robust method to define ADCC in this assay. Further studies analysing the role of monocyte-mediated ADCC are warranted.

## 2. Material and methods

### 2.1. Plasma samples

Plasma was collected from HIV+ subjects (n=53) and HIV- subjects (n=2). HIV+ subjects were receiving antiretroviral therapy at the time of recruitment. HIV+ subjects had a mean CD4 T cell count of 494 cells/μl (range 3–1360 cells/μl) and a mean plasma HIV RNA level of  $7.1 \times 10^4$  copies/ml, (range  $4 \times 10^1$ – $7.5 \times 10^5$  copies/ml) to reflect the spectrum of HIV disease states. All subjects provided written informed consent and the studies were approved by the relevant institutional ethics committees.

### 2.2. Purification of total IgG from plasma samples

Total IgG was purified from 300 μl plasma using Protein A IgG binding buffer (Thermo Fisher Scientific) and Protein A HP

MultiTrap plates (GE Healthcare) according to manufacturer's protocol with the following modifications: IgG binding was performed for 2 h at room temperature on a shaker, columns were not washed and elution was performed 3 times with elution buffer. Purified IgG was re-buffered in PBS and concentrated back to 300 μl using 30 kDa cut-off Amicon Ultra-4 filter units (Millipore).

### 2.3. Cells

CEM.NKr-CCR5 cells were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and maintained in RPMI; supplemented with 10% FCS and 1x Penicillin/ Streptomycin/ L-Glutamine (RF10) according to provider's protocol. PBMCs and NK cells were isolated from heparinized blood from healthy donors and monocytes from peripheral blood buffy packs provided by the Melbourne Red Cross Blood Bank. PBMCs were prepared by Ficoll density gradient centrifugation. NK cells were purified by negative selection using the RosetteSep NK cell isolation kit according to manufacturer's protocol (Stemcell Technologies). NK cell purity was above 90% detected by flow cytometry. Monocytes were purified by counter-current elutriation using a Beckman Coulter J-6 M/E centrifuge equipped with a JE-5.0 rotor, at 2200 rpm, 12 °C. Monocyte-containing fractions were collected and pooled. Monocyte purity was above 90% detected by flow cytometry.

### 2.4. RFADCC assay

The RFADCC assay was performed as previously described (Gómez-Román et al., 2006). Briefly,  $1 \times 10^6$  CEM.NKr-CCR5 cells in 100 μl of RF10 medium were coated with 3 μg of purified HIV<sub>AD8</sub> gp140 (clade B) (production of gp140 described elsewhere (Center et al., 2009)) for 1 h at room temperature. Uncoated CEM.NKr-CCR5 cells were treated identically but without adding gp140 protein. Coated and uncoated CEM.NKr-CCR5 cells were initially labeled with  $7.5 \times 10^{-7}$  M PKH26 (Sigma) solution for 4 min followed by addition of 400 μl FCS and 2 PBS washes. Cells were then stained with  $5 \times 10^{-8}$  M CFSE (Sigma) solution for 3 min followed by addition of 400 μl FCS. Cells were washed twice with PBS and once with RF10.  $2 \times 10^4$  PKH26+CFSE+ labeled CEM.NKr-CCR5 target cells in 20 μl were incubated (opsonized) with either 2 μl human plasma or human plasma-purified IgG for 30 min at 37 °C.  $2 \times 10^5$  PBMCs, NK cells or purified monocytes were added to target cells at a target to effector cell ratio of 1:10. Cells were incubated for 4 h at 37 °C and then stained for CD3 (anti-human CD3-PerCP, clone SK7), CD14 (anti-human CD14-APC-H7, clone MøP9) and CD56 (anti-human CD56-PECy7, clone NCAM-16.2) (all from BD Biosciences). After 30 min incubation at room temperature cells were washed once with PBS, fixed in 1% formaldehyde and analyzed using a FACS-Canto II cytometer and FlowJo analysis software (Version 9.4.9.). To assess CD107a mobilization in NK cells in specific experiments, Golgi Stop (5 μg/ml), BD Biosciences) and CD107a (anti-human CD107a-APC, BD Biosciences, H4A3) were added together with target cells.

### 2.5. Image stream flow cytometry

For image flow cytometry analysis, the RFADCC assay was performed as described above with the following minor modifications. For NK cell analysis CEM.NKr-CCR5 cells were double labelled with PKH26 and CFSE as above and PBMCs were stained for CD56 (CD56-Brilliant Violet, Biolegend, clone NCAM-16.2). For monocyte analysis CEM.NKr-CCR5 cells were single labelled with PKH26 and PBMCs were stained for CD14 (anti-human CD14-FITC, BD Biosciences, clone M5E2). All cells were co-stained with the nuclear stain DRAQ5 (1:200 final dilution, Invitrogen) before being fixed with 1% formaldehyde. Samples were acquired using the ImageStream 100 and analysed with IDEAS software (both Amnis). Single cells were identified using a bivariate plot of cell area vs aspect ratio (from the brightfield channel) and gating cells with an aspect ratio >0.7 and an area between 50 and 450 pixels. Due to the large size difference between the CEM cells and PBMC's, round clusters of PBMC's were a similar in size and aspect ratio to the CEM cells, so an additional gate was used to identify single cells. Using the spot count feature of the IDEAS software, the number of nuclei in each event was identified using the Draq5 nuclear stain, and events with single nuclei gated.

### 2.6. Deconvolution microscopy

For time course experiments using deconvolution microscopy, the RFADCC was performed as described above with the following modifications. CEM.NKr-CCR5 cells were labelled with PKH26 and PBMCs were stained for CD56 (CD56-Brilliant Violet, Biolegend, clone NCAM-16.2) and CD14 (anti-human CD14-FITC, BD Biosciences, clone M5E2) before target and effector cells were mixed together. At time zero DRAQ5 (Invitrogen) was added at a final dilution of 1:200 and cells were incubated at 37 °C/5%CO<sub>2</sub> using a Personal DeltaVision (Applied Precision) live cell imaging, deconvolution microscope. Images were taken after 10, 30, 90 and 120 min and deconvolved using SoftWorx Suite 2.0 (Applied Precision) and analysed using Imaris 3D software (Bitplane).

## 3. Results

### 3.1. Modified analysis and gating strategy using the RFADCC assay

The RFADCC assay has proved a very useful non-radioactive assay to measure ADCC, although in our hands the standard method of gating on PKH26 + targets cells and determining the proportion of CFSE-negative cells within this gate resulted in relatively high background levels of “killing” of non-antigen coated targets (Fig. S1) up to 20%. Although no information of background killing of uncoated target cells of previously published results is available, a mean background “killing” of 14.4% for HIV-pooled sera in the presence of gp120 has been reported (Gómez-Román et al., 2006). Following incubation of fresh PBMC and HIV + plasma with HIV-1 Env (gp140) coated CEM.NKr-CCR5 target cells for 4 h, we gated cells according to the published protocol for the RFADCC assay (Fig. 1A), in which PKH26 + CFSE- cells are considered to derive from

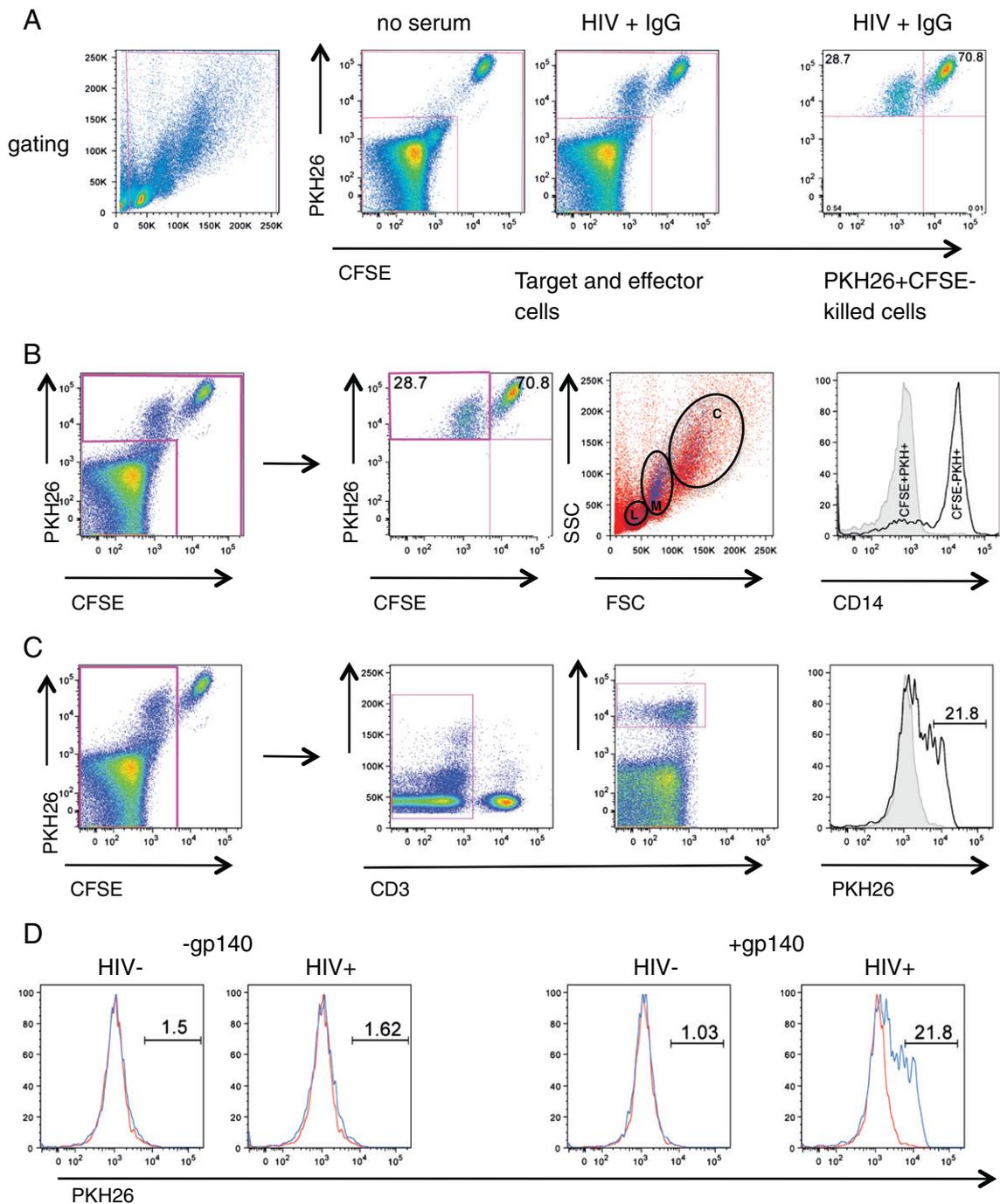
PKH26 + CFSE + target cells losing CFSE during early events of IgG-effector cell mediated cytolysis (Gómez-Román et al., 2006). In the example presented in Fig. 1, this resulted in an apparent killing of 28.7% of the target cells (Fig. 1A, right hand panel). Based on population characteristics shown in the bivariate CFSE/PKH26 dot-plot shown in Fig. 1A, centre right panel, PKH26 + CFSE- cells clearly emerge from the PKH26-CFSE- pool of PBMC/effector cells. This prompted us to explore the origin of the CFSE-PKH + cells by back-gating the PKH26 + CFSE-population onto the whole population in the forward (FSC) and side scatter (SSC) plot. Although a few of the PKH26 + CFSE- cells were located within the lymphocyte and CEM.NKr-CCR5 gate the majority of PKH26 + CFSE- cells fell within the characteristic monocyte area (Fig. 1B, second right). Staining with CD3 and CD14 antibodies showed that the PKH26 + CFSE- cells were also CD3-CD14 + (Fig. 1B right), confirming their monocyte origin. Importantly, none of the PKH26 + CFSE + target cells were positive for CD14, showing that monocytes do not phagocytose whole unkilld target cells within the time frame of the assay.

To determine the role of monocytes in the apparent killing of target cells we gated on all CFSE- cells (Fig. 1C) and then on CD3- and CD14+ cells within this population (Fig. 1C, centre panels). The proportion of monocytes positive for PKH26 was then determined (Fig. 1C, right). The ADCC effector activity in this gating strategy is defined as the percent of PKH26 + CD3-CD14+ monocytes above the no antibody control. Using this new CD14 gating strategy we showed that the emergence of PKH26 + CD3-CD14+ monocytes was dependent on the presence of HIV gp140 Env protein and plasma IgG from HIV + subjects (Fig. 1D).

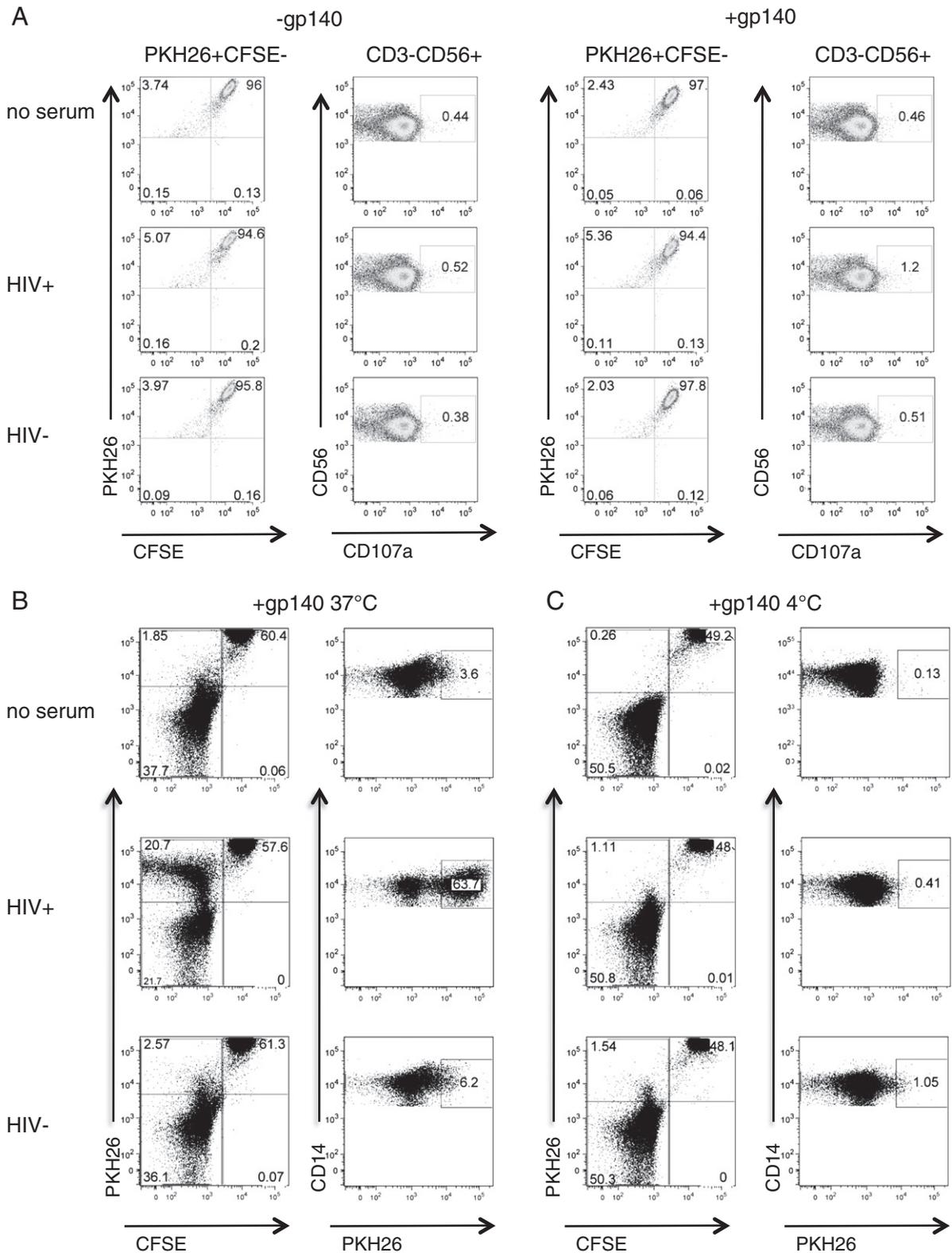
### 3.2. RFADCC assay using purified NK cells and monocytes as effector cells

The above results suggested that monocytes play a major role in the RFADCC assay, but it is unclear whether they kill target cells directly or phagocytose cells and/or cellular debris killed by NK cell ADCC activity. To address this we cultured NK cells purified by negative selection (>90% purity) or monocytes purified by countercurrent elutriation (>90% purity) with PKH26 + CFSE + CEM.NKr-CCR5 gp140-coated target cells in the presence and absence of HIV + serum. PKH26 + CFSE- cells were not detected when gp140-coated targets were cultured with purified NK cells in the presence of HIV + plasma with known ADCC activity (Fig. 2A). Consistent with a lack of apparent killing, there was no detectable degranulation, as assessed by CD107a staining, in NK cells in the presence of HIV + plasma and gp140.

In contrast, PKH26 + CFSE-cells were detected in the presence of gp140-coated targets and HIV + plasma (Fig. 2B, left panel). The CD3-CD14+ monocytes acquired PKH26 from gp140-coated target cells in the presence of HIV + plasma but not in the presence of HIV- plasma or in the absence of any serum (Fig. 2B right panel). Acquisition of PKH26 staining by monocytes was abolished when the assay was conducted at 4 °C suggesting that it is due to internalization and not attachment of labeled cells to the monocyte surface (Fig. 2C). Overall these results confirm that monocytes, rather than NK cells, play a major role in the RFADCC assay.



**Fig. 1.** ADCC responses of HIV + purified IgG using the RFADCC assay. A) standard gating strategy on PKH26 + population according to Gomez-Roman et al (Gómez-Román et al., 2006) detecting CFSE loss in target cells when gated on PKH26 + cells compared to gating on target (PKH26 + CFSE+) and effector cells (PKH26-CFSE-) identifying the emergence of CFSE-PKH26 + population from the PBMC. B) Demonstration of origin of the PKH26 + CFSE- cells using a back-gating strategy into forward scatter (FSC) and sideward scatter (SSC) plots (L: lymphocytes; M: monocytes; C: CEM.NKr-CCR5) and additional staining for CD14 expression. C) The role of monocytes in target killing. The new analysis/readout is performed by gating on CFSE- cells followed by gating on CD3-CD14 + monocytes and analysis of PKH26 expression for these monocytes. Side by side comparison of PKH26 expression for CD3-CD14 + monocytes in the absence of HIV + plasma (gray filled histogram) and presence of HIV + plasma (black open histogram). D) Confirmation that PKH26 signal for CD3-CD14 + monocytes is specific for gp140 coated CEM.NKr-CCR5 target cells (gp140 +) in the presence of HIV + IgG. Red histograms represent the no plasma control and blue histograms represent the respective plasma.



**Fig. 2.** Measurement of ADCC responses in the presence of HIV + plasma for A) purified NK cells; gating on either PKH26 + cells (loss of CFSE) or on CD3-CD56 + cells (CD107a degranulation) and B) monocytes; gating on either PKH26 + cells (loss of CFSE, left panel) or CD3-CD14 + cells (PKH26 expression, right panel) at 37 °C. C) To distinguish between internalization and binding of PKH26 + targets using monocytes the experiments were also performed at 4 °C.

### 3.3. Interaction of monocytes with target cells by Image flow analysis

Monocytes are largely known for their ability to engulf and phagocytose target cells. From the above experiments, it was unclear if monocytes were simply ingesting whole, opsonized target cells or ingesting fragments of cells following killing by other mechanisms. To further investigate the monocyte-target cell interaction we performed the RFADCC assay and imaged monocytes, NK cells and CEM.NKr-CCR5 target cells in the presence of gp140 and HIV + plasma by image flow cytometry. The "brightfield aspect ratio" (measures the ratio of the cell's minor axis to its major axis which is close to 1 for round cells) was plotted against the "brightfield cell area" to identify single cells. Due to the large size difference between the CEM cells and PBMCs, single events were further distinguished by single nuclei using the spot count feature in the Draq5 channel (nuclei stain) (Fig. 3A1 and B1). Singlets were plotted on bivariate CD14 + PKH26 + and singlet CD56 + PKH26 + dot plots. Images from CD14 + PKH26 + monocytes showed that PKH26 staining of monocytes is not due to bound target cells but suggest that monocytes had internalized PKH26 + membrane rather than phagocytosed whole target cells as PKH26 stain appears in endocytic/phagocytic vesicles (Fig. 3A2). In contrast to monocytes, NK cells were not observed to take up the PKH26 dye (Fig. 3B2).

### 3.4. Uptake of PKH26 + membrane by monocytes without phagocytosis

Imaging flow cytometry showed distinct uptake by normal sized monocytes of the PKH26, but due to the relatively low resolution the possibility of phagocytosis could not be completely excluded. To further examine the mechanism of cell killing and exclude the possibility of phagocytosis of whole target cells, we analyzed the fate of target cells over time in the RFADCC assay using high resolution deconvolution microscopy. PBMC were cultured with PKH26 + CFSE + gp140-coated CEM.NKr-CCR5 target cells in the presence of HIV + plasma. Within 10 min we observed physical attachment of CEM.NKr-CCR5 target cells to CD14 + monocytes that persisted over time (Fig. 4). From 30 min onwards attachment of monocytes to CEM.NKr-CCR5 target cells was associated with formation of dense PKH26-staining bodies. After 60 min we observed that monocytes associated with target cells showed punctuate staining with PKH26 similar to that of the target cells. There was no evidence of phagocytosis of entire target cells (Fig. 4).

### 3.5. Analysis of RFADCC assay in 53 HIV + human samples

The above experiments show that in the RFADCC assay, PKH26 dye derived from opsonised target cells becomes

associated with monocytes. This suggested that an alternate method to analyze ADCC activity in the RFADCC assay would be to gate on monocytes taking up the PKH26 dye. To validate the use of a monocyte gating strategy, and to confirm that our initial observations with plasma were not mediated by complement activation, we tested purified IgG from plasma of 53 HIV-infected subjects and PBMC from three uninfected individuals in the RFADCC assay. We compared the standard gating on the PKH26 + population and the loss of CFSE ("CFSE") with the new PKH26 + CD3-CD14 + readout ("CD14") (Fig. 5). We detected significant background killing of target cells in the absence of gp140 using the standard approach of analysis (PKH26 + CFSE-, mean 17.81%, range 6.3–39.77). However, there was little or no background using our CD14 based gating analysis (PKH26 + CD3-CD14+, mean 1.25%, range 0.31–7.56) (Fig. 5A). To obtain the net ADCC response (Fig. 5C) we subtracted the background measured for target cells without gp140 (Fig. 5A) from the response measured for gp140 coated target cells (Fig. 5B). The overall ADCC response ("net ADCC") was higher for the standard analysis of PKH26 + CFSE- cells compared to the CD14 gating analysis with mean killing of 18.49% (2–35.84) and 12.65% (7.25–17.89), respectively (Fig. 5C). Using the PKH26 + CD3-CD14 + readout for the RFADCC assay, a higher signal to noise ratio of gp140-coated and gp140-uncoated (background) cells was detected compared to the standard PKH26 + CFSE- analysis (Fig. 5D).

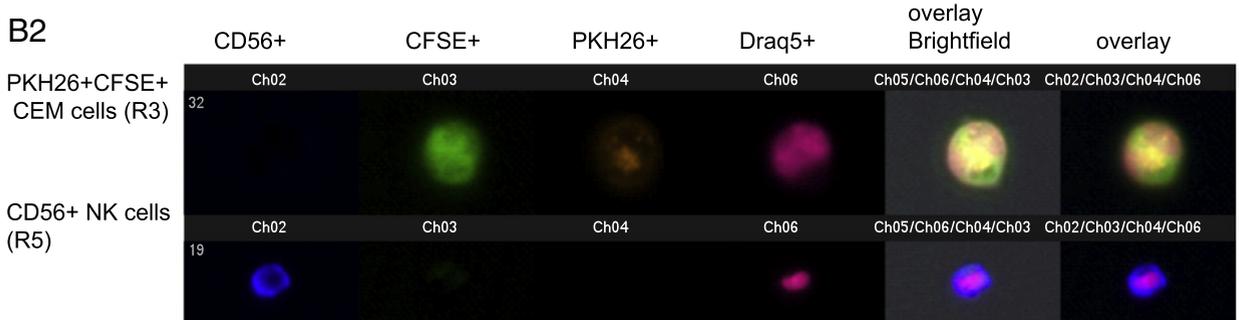
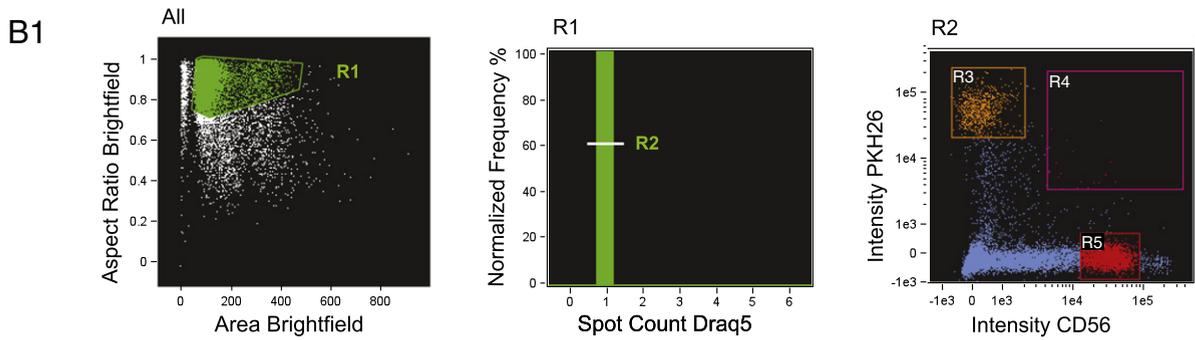
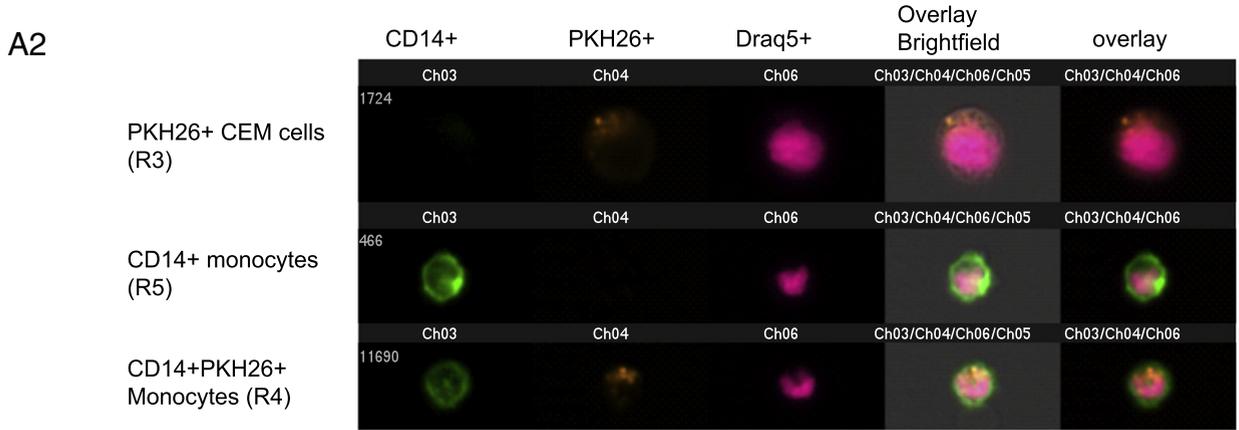
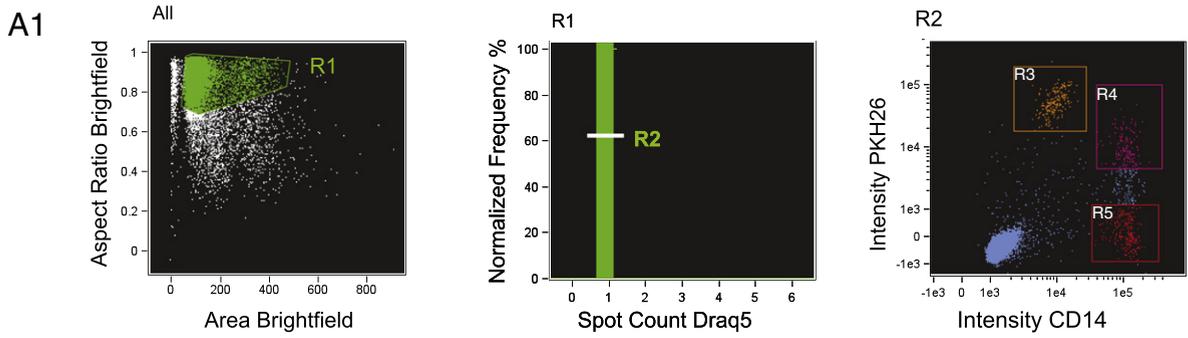
There is typically some variability in the killing mediated by NK cells from different healthy donors. To ensure that the results were valid across multiple sources of healthy donor effector cells, we tested fresh PBMC from three subjects against the entire panel of 53 HIV + IgG samples. Fig. 5E shows the "net ADCC" response which was different for both the standard CFSE- and CD14 readout but similar magnitudes of responses were obtained for all three donors.

## 4. Discussion

ADCC is an immune response of emerging interest with a potential role in controlling HIV infection (Lambotte et al., 2009; Berger and Alter, 2011; Chung et al., 2011). ADCC activity is most commonly attributed to NK cells although monocytes and granulocytes also bear Fc $\gamma$  receptors (Fc $\gamma$ R), including Fc $\gamma$ RI, Fc $\gamma$ RIIa/b and Fc $\gamma$ RIII and have been shown to kill targets via ADCC (Barker and Reisfeld, 1993; Horner et al., 2007; Wu et al., 2008). Effector functions of monocytes/macrophages focus mainly on phagocytosis with an increasing interest in antibody-dependent phagocytosis in the context of HIV (Ackerman et al., 2011; Dugast et al., 2011; McAndrew et al., 2011).

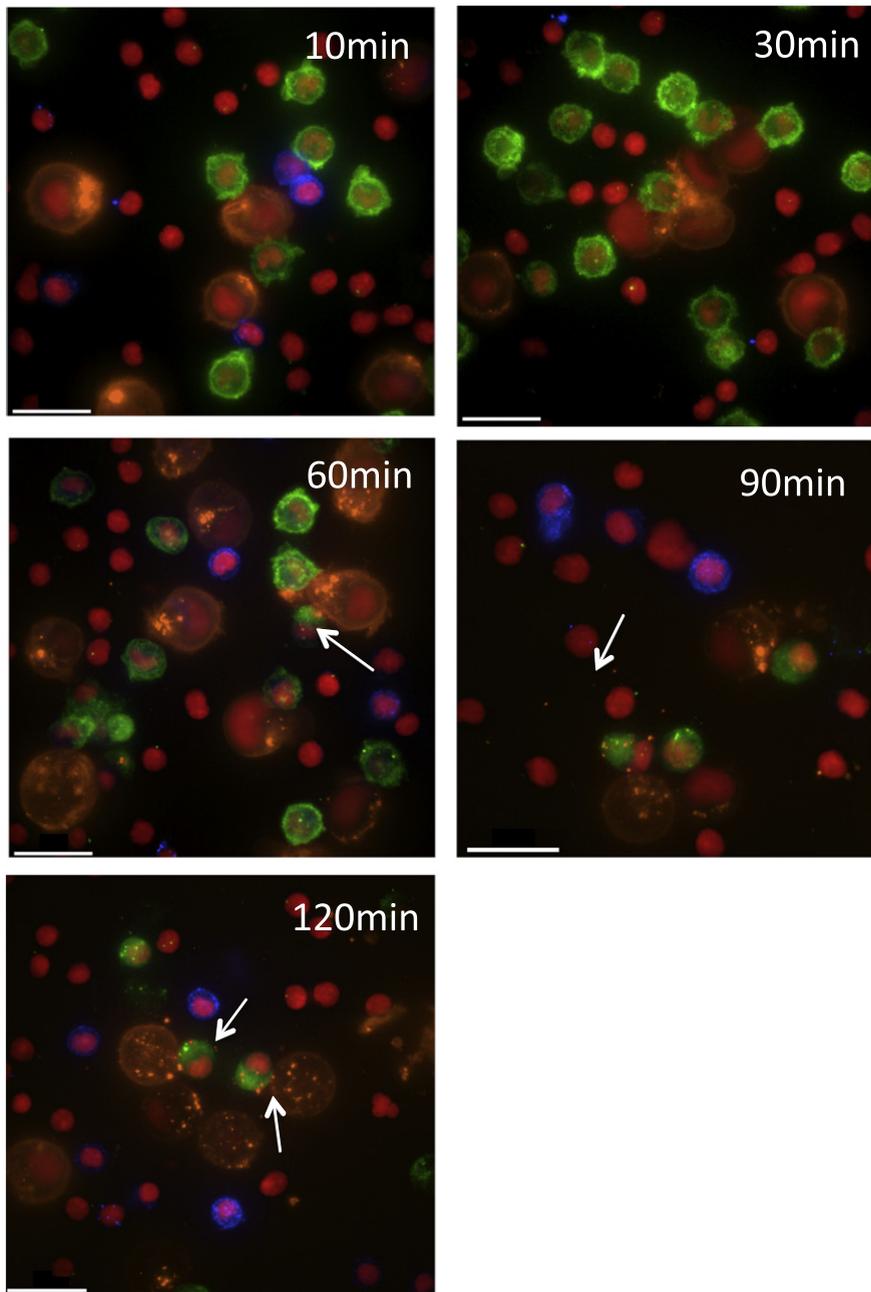
The RFADCC assay has become a widely used tool to study NK cell ADCC. We demonstrate that NK cell activity is not the major killing mechanism detected in the RFADCC assay if PBMCs are used as effector cells. We performed the RFADCC with purified NK cells and were unable to detect significant degranulation in the presence of HIV + IgG and gp140.

**Fig. 3.** Image stream flow cytometry analysis after RFADCC assay using healthy PBMC and HIV + plasma. A1) Single cells were identified by plotting cell area vs aspect ratio (R1) and distinguishing events with single nuclei using the spot count feature in the Draq5 channel (R2). Singlet CD14 + monocytes and PKH26 + CEM.NKr-CCR5 cells were plotted on CD14/PKH16 dot plots (R3–5). A2) representative images for cell controls CD14 + monocytes (R5) and PKH26 + CEM.NKr-CCR5 cells (R3) and CD14 + PKH26 + double positive monocytes (singlets) (R4) are shown. B1) Single cells were identified by plotting cell area vs aspect ratio (R1) and distinguishing events with single nuclei using the spot count feature in the Draq5 channel (R2). Singlet CD56 + NK cells and PKH26 + CFSE-CEM.NKr-CCR5 cells were plotted on CD56/PKH16 dot plots (R3–R5). B2) representative images for cell controls CD56 + NK cells (R5) and PKH26 + CEM.NKr-CCR5 cells (R3) are shown. DRAQ5 was used for co-staining of the nuclei.



However this could be due to NK cell activation during the purification process or because the target:effector ratio is not ideal. Further evaluation of the RFADCC with purified NK

cells or NK cell lines is warranted to confirm that the RFADCC assay can measure NK cell-mediated ADCC. In fact our data indicates that the RFADCC assay does not measure killing by

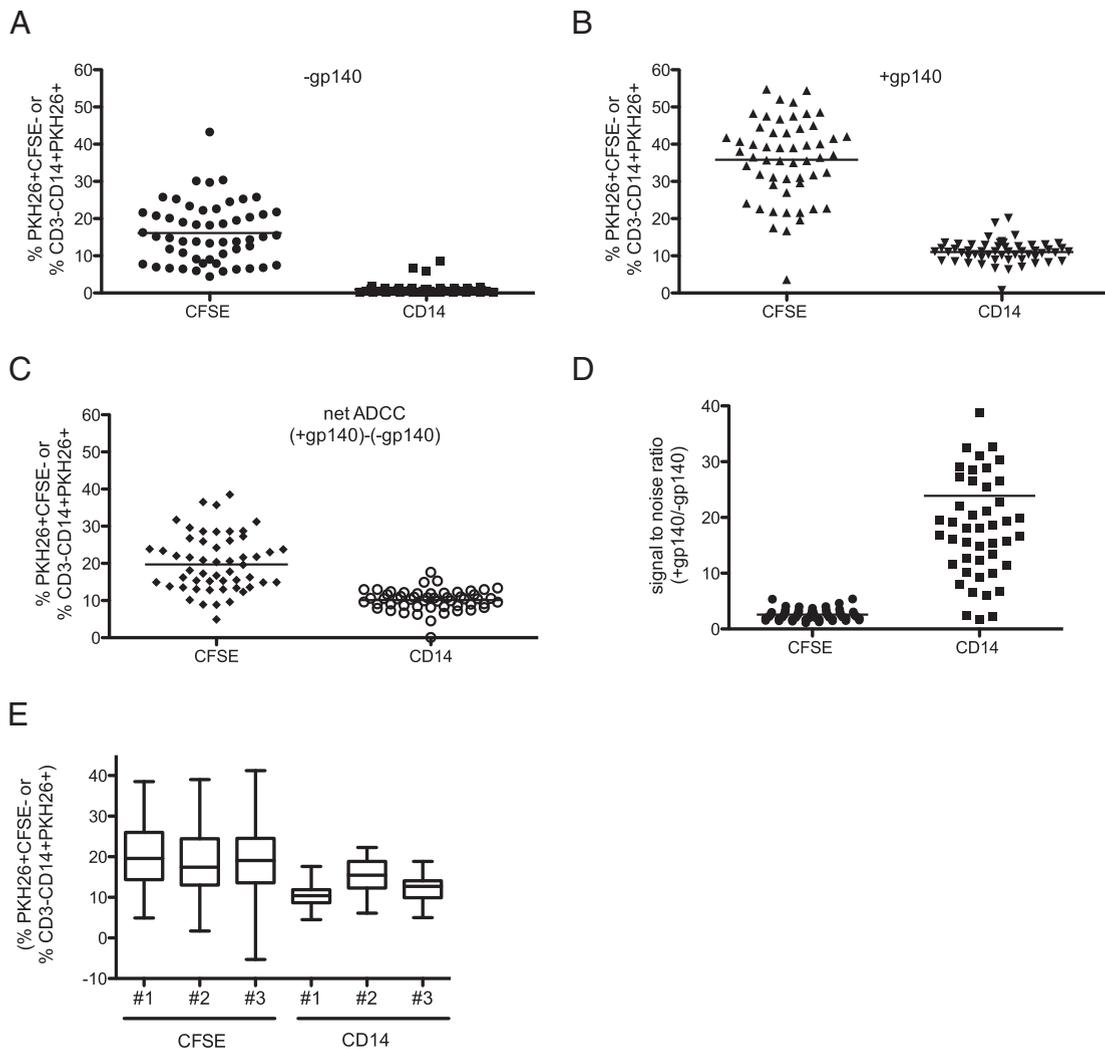


**Fig. 4.** Deconvolution microscopy assessing interaction of monocytes and NK cells in the RFADCC assay over time. Cem.NKr-CCR5 cells were labelled with PKH26 (orange), monocytes with CD14-FITC (green) and NK cells with CD56-Brilliant violet (blue). Nuclei were co-stained with DRAQ5. Arrows indicate PKH26+ membrane uptake by monocytes. Scale bars indicate a size of 20  $\mu$ m.

NK cells but rather measures IgG-mediated monocyte function. The PKH26+CFSE- cell population typically reported as “killed” target cells in this assay are actually CD3-CD14+ monocytes with internalized PKH26 membrane fragments, rather than CEM.NKr.CCR5 target cells that have lost CFSE. Moreover, our microscopy results clearly show that monocytes take up PKH26-stained target cell membrane without evidence of characteristic for classical phagocytosis of the intact target cell. Our microscopy results showed that

membranes of monocyte-engaged target cells display distinct membrane vesicles that could be apoptotic bodies passed on to the monocytes. Further experiments are required to elucidate the precise mechanisms of how and when the target cells are killed during this process.

Previous studies have reported monocyte-mediated non-phagocytosis effector function in the context of malaria and cancer using either antibody-dependent cellular inhibition or  $^{51}\text{Cr}$  release assays (Shaw et al., 1978b, 1978a; Khusmith and



**Fig. 5.** Comparison of the standard analysis (CFSE) and the monocyte analysis (CD14) using 53 IgG samples purified from plasma of HIV-infected individuals. Mean % of PKH26 + CFSE- cells in comparison to mean % of CD3-CD14 + PKH26 + cells in the absence of gp140 (background) (A) and presence of gp140 (B). Mean % of PKH26 + cells for both readouts was plotted after subtraction of the background ["net ADCC"] (C). Comparison of signal to noise ratio [+gp140/-gp140] for both readouts (D). Results for A-D were obtained using PBMC from one healthy donor and 53 HIV + IgG samples. The "net ADCC" response (% of either PKH26 + CFSE- or PKH26 + CD3-CD14 + cells) using PMBC from three healthy donors (#1- #3) and 53 HIV + IgG samples (E).

Druilhe, 1983; Lunel and Druilhe, 1989; Bouharoun-Tayoun et al., 1990, 1995). Reported anti-malaria activity mediated by monocytes in the presence of immune IgG, which is distinct to phagocytosis, suggested an ADCC-like mechanism through monocyte activation and release of soluble mediators, like TNF, leading to killing of the parasite (Khusmith and Druilhe, 1983). Jafarshad et al further demonstrated that the malaria-specific ADCC mechanism is similar to ADCC mechanisms described for other pathogens. However this parasite-directed ADCC requires Fc $\gamma$ RIII and Fc $\gamma$ RIIa but not Fc $\gamma$ RI, which is only required for phagocytosis (Jafarshad et al., 2007).

NK cell-mediated HIV-specific ADCC is likely a key effector mechanism against primary blood cells expressing HIV antigens. We and others have shown NK cell activation by intracellular cytokine staining when PBMC are cultured with HIV + serum and HIV antigens (Stratov et al., 2008; Chung

et al., 2009; Tiemessen et al., 2009). However, the RFADCC assay using the CEM.NK $\gamma$ -CCR5 T-cell line is clearly dependent on monocyte activity. The CEM.NK $\gamma$ -CCR5 cell line coated with gp140 Env protein may present antigen to antibodies differently compared to HIV-infected or coated primary cells. This may in turn result in more efficient activation of monocytes rather than NK cells. Another possibility is the absence of cytokines in the RFADCC assay which are necessary for efficient NK cell killing as previously shown in the context of cancer (Wu et al., 2008). CEM.NK $\gamma$ -CCR5 cells are resistant to natural NK cell killing probably due to the lack of co-stimulatory interactions which could also have an impact on ADCC-mediated NK cell killing. Interestingly, we previously observed a poor correlation between the RFADCC and NK cell activation assays (Stratov et al., 2008; Chung et al., 2009). This may reflect the involvement of different effector cells in the different assays. It would be interesting to also examine

monocyte activation in other ADCC assays to further probe monocyte-mediated ADCC activities. Results obtained from assays using infected cells (Pollara et al., 2011) suggest that NK cells play an important role in HIV ADCC. However the involvement of monocytes in these assays has not been studied.

We have studied IgG samples from HIV-infected subjects with varying stages of disease. It would be of interest to evaluate IgG or serum samples in the context of vaccination studies. Others have detected ADCC responses after vaccination using the RFADCC assay (Gómez-Román et al., 2005; Chung et al., 2009; Florese et al., 2009; Vaine et al., 2010; Xiao et al., 2010; Patterson et al., 2011); whether these responses also reflect monocyte-mediated ADCC remains to be shown.

## 5. Conclusion

Our studies clarify the understanding of the cellular events underlying IgG-mediated HIV-specific cellular effector function detected by the RFADCC assay. We demonstrate that this assay primarily reflects Ab-mediated monocyte function and not NK cell ADCC and therefore has to be treated with caution in regards to the interpretation for NK cell-mediated ADCC. Presumably, lysis is not mediated by complement activation because the effect is seen with purified IgG. Our amended gating on monocytes taking up PKH26 membrane reduces the background killing signals remarkably. This should be a useful improvement of the assay when ADCC responses are expected to be low, as in the case of early responses following vaccination.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2012.07.006>.

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