Author/s:
Ana-Sosa-Batiz, F;Johnston, APR;Liu, H;Center, RJ;Rerks-Ngarm, S;Pitisuttithum, P;Nitayaphan, S;Kaewkungwal, J;Kim, JH;Michael, NL;Kelleher, AD;Stratov, I;Kent, SJ;Kramski, M

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HIV specific antibody-dependent phagocytosis (ADP) matures during HIV infection

Fernanda Ana-Sosa Batiz[1*], Angus P.R. Johnston [2], Haiyin Liu[2], Rob J. Center [3], Supachai Rerks-Ngarm[4], Punnee Pitisuttithum[5], Sorachai Nitayaphan[6], Jaranit Kaewkungwal[7], Jerome H. Kim[8], Nelson L. Michael[8], Anthony D Kelleher[9], Ivan Stratov[1], Stephen J. Kent[1*] and Marit Kramski[1]

[1] Department of Microbiology and Immunology at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne, Australia

[2] Drug Delivery, Disposition and Dynamics
Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia

[3] Burnet Institute, 85 Commercial Road, Melbourne, Australia

[4] Department of Disease Control, Ministry of Public Health, Bangkok, Thailand

[5] Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

[6] Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

[7] Data Management Unit, Mahidol University, Bangkok, Thailand

[8] U.S. Military HIV Research Program, Walter Reed Army Institute of Research, Rockville, USA

[9] The Kirby Institute for Infection and Immunity in Society, UNSW Medicine, Sydney, Australia

*Corresponding author:

Prof Stephen Kent

Department of Microbiology and Immunology, at the Peter Doherty Institute for Infection and Immunity

University of Melbourne

792 Elizabeth Street

phone: +61 (0) 3 83449939

fax: +61 (0) 3 83443846

skent@unimelb.edu.au

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Antibody-dependent phagocytosis (ADP) is a potentially important immune mechanism to clear HIV. How HIV-specific ADP responses mature during HIV infection or in response to vaccinations administered, including the partially successful RV144 HIV vaccine, is not known.

We established a modified ADP assay to measure internalisation of HIV antibody-opsonised targets using a specific hybridisation internalisation probe. Labelled beads were coated with both biotinylated HIV gp140 envelope protein and a fluorescent internalisation probe, opsonized with antibodies and incubated with a monocytic cell line. The fluorescence derived from the fluorescent internalisation probe on surface bound beads, but not from internalised beads, was quenched by the addition of a complementary quencher probe.

HIV Env-specific ADP was measured in 31 subjects during primary infection and early chronic HIV infection. Although ADP responses were present early during HIV infection, a significant increase in ADP responses in all 31 subjects studied was detected ($p< 0.001$). However, when we tested 30 HIV-negative human subjects immunised with the Canarypox/gp120 vaccine regimen (subjects from the RV144 trial) we did not detect HIV-specific ADP activity.

In conclusion, a modified assay was developed to measure HIV-specific ADP. Enhanced ADP responses early in the course of HIV infection were observed but no ADP activity was detected following the vaccinations administered in the RV144 trial. Improved vaccine regimens may be needed to capitalise on ADP-mediated immunity against HIV.

3-6 Key words (in alphabetical order): antibody-dependent phagocytosis (ADP), human immunodeficiency virus (HIV), specific hybridisation internalisation probe (SHIP)

Introduction

Antibodies (Abs) are directed against multiple HIV epitopes {Alter:2010il} and generally develop within a few weeks after infection. Neutralizing Abs directed against the HIV envelope protein can prevent HIV infection in vitro and prevent infection of macaques with SIV/HIV-Env chimeric viruses {Hessell:2009ig, Parren:2001wc, Mascola:1999wk, Moog:2013ht}. The development of neutralizing Abs able to block infection of a broad range of HIV strains requires a long Ab maturation process which usually takes years to develop {Stamatatos:2009ki}. HIV broadly neutralizing Abs are however difficult to elicit by standard vaccination regimes as they are unable to recapitulate the prolonged presence of antigen required for generating highly affinity matured broadly neutralizing Abs {Haynes:2012ba}.

Although neutralizing Abs are considered an efficient mode of Ab-mediated protection against HIV, Fc-mediated immune functions also play an important role in protection {Hessell:2007fg, Hezareh:2001ew}. In contrast to the prolonged development of broadly neutralizing Abs, all HIV-infected individuals rapidly develop non-neutralizing Abs during the primary phase of their infection. Abs can mediate different effector functions through the binding of their Fc region to the Fcγ-receptor (FcγR) which include antibody-dependent phagocytosis (ADP) and antibody-dependent cellular cytotoxicity (ADCC) {Forthal:2009jp, Huber:2007hy}.

Numerous studies now support the role for ADCC activity in the control of HIV infection where the presence of ADCC correlates with a better disease prognosis and slow HIV progression {Lambotte:2009bm, Baum:1996wo, Ahmad:2001ua, Johansson:2011cq, Wren:2013ge, Chung:2011jh}. In comparison to neutralizing antibodies, ADCC Abs develop much quicker and require lower levels of Ab maturation {Bonsignori:2012ub}.

In contrast to the growing body of literature on ADCC antibodies, comparatively little is known about ADP-mediating Abs in HIV infection. ADP is a potentially important Ab effector function to eliminate immune complexes (i.e. opsonized HIV) and HIV-infected cells. It is unknown when ADP Abs arise during HIV infection and if they need a similarly long maturation process as neutralizing Abs. It was previously shown that ADP-mediating Abs from controllers and untreated progressors exhibited similar ADP activity which was also elevated compared to subjects on anti-retroviral therapy {Ackerman:2013ee}. However, little is known about ADP during early HIV infection.

The role of ADP antibodies in protection from HIV is also of growing interest. Barouch et al recently studied ADP in a macaque vaccination study using Adenovirus/poxvirus and adenovirus/ adenovirus vectors expressing HIV-1 mosaic antigens. Using this vaccination strategy they demonstrated that protection was correlated with functional non-neutralizing ADP Abs {Barouch:2013jv}. However, this is the only study that investigated the potential of vaccine-induced HIV-specific ADP Abs in preventing HIV infection. In humans, the partially protective RV144 vaccine elicited minimal HIV-specific neutralizing Abs but did induce non-neutralizing Abs {RerksNgarm:2009bk}. Post hoc analyses demonstrated that ADCC was associated with protection from HIV acquisition in the RV144 trial {Haynes:2012ka} and that serum IgA elicited by the vaccine interfered with binding and functional activity of ADCC-mediating serum IgG {Tomaras:2013fm}. However, it is unknown if non-neutralizing Abs with ADP function were also induced by the RV144 vaccine regimen.

To study the role played by Abs on clearance by phagocytosis of virus infected cells it is necessary to use a robust method able to evaluate ADP in vitro. The standard ADP assay developed by Ackerman et al assesses the phagocytic potential of Abs using Env protein coated fluorescent beads that are opsonized with purified IgG and subsequently incubated

with the phagocytic cell line THP1, which expresses all 3 main FcgRs {Ackerman:2011eo, McAndrew:2011ip}. A phagocytic score (% bead positive cells x mean fluorescence intensity (MFI)) is calculated as an indicator of ADP. However, this assay has the measures the sum of binding of beads on the cell surface in addition to truly phagocytosed (i.e. internalised) beads. To specifically assess the ability of antibodies to assist internalization of beads, we adapted a recent published method by *Liu et al* to study internalized beads {Liu:2013um}. The assay involves adding a specific hybridization internalisation probe (SHIP) that allows truly phagocytised beads to be distinguished from surface bound beads (Fig. 1). We used this assay to investigate the development of ADP-mediating Abs during the early stages of HIV infection and to determine if they can be induced by the RV144 preventative vaccination strategy.

Results

ADP-SHIP assay reliably discriminates between surface-bound and internalised ADP targets.

Non-neutralizing Ab responses may assist in immunity to HIV and there is a need to develop ADP assays to specifically measure HIV antibody-specific phagocytosis. The standard ADP assay published {Ackerman:2011eo} utilises beads with only one colour. Flow cytometry is used to determine the % of cells associated with beads but it is not possible to distinguish between internalized beads and surface-bound beads (Fig 1A).

We developed a modified ADP assay, called ADP-SHIP, which utilises both FITC and Cy5 (fluorescent internalisation probe, FIP_{Cy5}) labelled beads. This dual labelling allows for the discrimination of surface bound and internalised beads after the addition of a complementary quenching DNA sequence (QP_C) at the end of the assay. The QP_C cannot diffuse through the membrane of the cell and therefore it only binds to FIP_{Cy5} on beads

bound to the cell surface resulting in the quenching of the Cy5 fluorescence (Fig 1B). Surface bound and internalized beads are easily detected by flow cytometry as FITC+ single-positive and FITC+Cy5+ double-positive cells, respectively. All FITC+ cells are analysed for their Cy5 fluorescence (gating shown in Figure 2A). Figure 2B shows that the additional binding of the FIP_{Cy5} does not diminish the overall % of beads positive cells. This was independent of whether gp140 was bound before, after, or simultaneously with FIP_{Cy5} (Fig S1A). It further showed that for IgG from healthy donors the % of phagocytosed beads is overestimated as a proportion of the beads are only bound to the surface of the cells. An advantage is that the ADP-SHIP assay directly determines the % of cells which have internalized beads (i.e. are double positive FITC+FIPCy5). In addition, the number of beads phagocytosed by each cell (one per cell, 2 per cell, or more than 2 per cell) can be reliably determined. Note that there is a low level of internalization of gp140-coated beads in the absence of HIV Abs and thus the ADP-SHIP assay assesses the increase in phagocytosis above this level (Fig 2B). Discrimination of surface-bound and internalised beads as seen by flow cytometry was confirmed by deconvolution microscopy (Fig 2C).

Similar to the standard ADP assay, the ADP-SHIP assay needs optimal conditions for each gp140 protein used including (i) gp140 protein concentration used for binding to the beads (Fig S1B), (ii) degree of gp140 biotinylation (Fig S1D) and (iii) IgG concentration (Fig S1B). These factors were carefully optimised (shown in Figure S1). The optimal levels of gp140 protein coating were 0.5 μg/μl beads, the degree of biotinylation was 20-fold biotin excess and the concentration of IgG employed in the assay was 1 μg/ml. Coating of the beads with gp140 did not lead to an increase in binding of the beads to the surface of the target cell (Fig S1F).

To validate the new ADP-SHIP assay we assessed ADP activity in 20 HIV-infected individuals and compared it to ADP activity of 20 healthy donors. Both the standard ADP

and ADP-SHIP assays allowed for discrimination between HIV+ and HIV- ADP activity. It is difficult to directly compare these two assays due to the different readouts, however the difference in the ratio of phagocytosis of HIV-positive and HIV-negative samples was 2.08 for the standard ADP assay and 2.04 for the ADP-SHIP assay (ratio of either the mean phagocytic score or % FITC+Cy5+ cells of HIV+ /HIV- samples) (Fig 3A). However, we did note that the ADP-SHIP assay had a wider dynamic range compared to the standard assay. There was no difference in the levels of surface-bound gp140-coated beads between the HIV- and HIV+ samples (Fig 3B). The coefficient of variation of the ADP-SHIP and standard ADP assay were similar at 4.56% and 7.24% respectively across 10 replicates (Fig S1G). When the number of beads per cell was analysed, the ADP-SHIP assay revealed that the majority of cells had only phagocytosed one bead per cell (mean: 14.1% of all cells; range 5.4%-19.5%) but also a large proportion of one surface bound bead per cell (mean: 6.3%; range 4.9%-10%) was detected. A smaller proportion of cells had two (mean 5.9%, range 1.2%-9.8%) or more than two beads per cell (mean 4.1%; range 0.47%-8.7%) phagocytosed (Fig 3B). Almost none of the cells had two or more than two surface-bound beads per cell (mean: 1.1%; range 0.6%-3.5%). For this analysis it is not necessary to use microscopy to detect the number of internalised beads. Through flow cytometry it is possible to detect the whole cell and not only slices through the cell, giving the possibility of determining the number of beads internalised per cell.

ADP antibodies during early HIV infection increase over time

Little is known about the development of ADP Abs during the course of HIV infection. We therefore determined ADP in HIV-infected patients (n=31) who were not on treatment during primary and early chronic infection. Serum samples were paired and median duration of primary and early chronic infection was 4 months and 25 months, respectively.

All subjects displayed ADP Abs at the early time point, which was well above the ADP signal for HIV-ve subjects (Fig 4A). Over the 2-year follow up, ADP activity increased significantly in 30 of the 31 individuals. For primary and early chronic infection samples there was a respective median ± IQR ADP activity of 14.31% ± 7.20 and 25.19% ± 6.28 % FITC+Cy5+ cells. When the data was broken down by the number of internalised beads per cell (1, 2 or > 2), we found an increase in ADP across all subgroups in the early chronic infection samples (Fig 4B). There was a significative increase in the number of cells with 1 internalised bead for early chronic infection samples (median: 15.61%; range: 11.54% - 20.55%) compared to primary infection (median: 9.93%; range: 7.21% – 18.14%). The number of cells with more than 1 bead internalised was also increased (median for primary infection samples: 4.59%; range: 2.85% - 13.16%; median for early chronic infection samples: 9.82%; range: 5.37% -19.10%). Cells with 1, 2 or >2 beads only bound to the surface of the cell and was independent of primary and early chronic infection (Fig 4C). Since there was a range of time points at which the samples were collected (from 1-34 months after the estimated time of HIV infection), we plotted the duration of infection against the level of ADP. We found ADP Abs were correlated with the duration of infection (Fig 4D).

No detectable ADP antibodies in RV144 vaccinees.

The ADP-SHIP assay was used to assess ADP activity in HIV vaccinees of the RV144 trial {RerksNgarm:2009bk} in order to gain insight into whether ADP can be elicited by preventative vaccines and to dissect their potential role in protection. Thirty samples from vaccine recipients and 30 samples from placebo recipients were kindly provided by the Thai-MHRP, all 2 weeks after the final vaccination (week 26 of the trial). The IgG from plasma was first tested for ADP activity against Env gp140 subtype B (AD8), which was

one of the 2 subtypes used for the protein boost used in the vaccination trial. Using the ADP-SHIP assay no significant difference between vaccinees and placebo could be detected (Fig 5A). Vaccine and placebo samples from the RV144 study did not show ADP activity above the HIV-negative sample and were well below the HIV+ control samples. The RV144 vaccine regimen also employed a protein boost with a subtype A/E Env protein. All samples were therefore also tested for their ADP activity against Env gp140 subtype A/E 966, which was the same subtype used for the vaccine DNA prime. No significant difference could be detected in ADP activity between placebo and vaccine recipients using either 966 or AD8 gp140 (Fig 5B). There was also no differences in levels of surface bound beads across the 3 groups for either the subtype B or A/E gp140 protein coated beads (Fig 5C,D).

Discussion

There is limited knowledge about the potentially important role of ADP Abs in the control or prevention of HIV infection. Such a role is supported by (i) the correlation of FcγR2a polymorphisms with HIV disease progression {Forthal:2012ux}, (ii) the correlation of the presence of ADP-mediating IgG2 (binding more efficiently to FcgRIIa) with delayed progression {French:2010bj, NgoGiangHuong:2001cs} and (iii) HIV-associated down-regulation of FcγR2 expression in progressive infection leading to reduced ADP activity {Dugast:2011gj}.

To standardize the measurement of ADP-mediating Abs Ackerman et al developed an ADP-assay which employs a widely utilized monocytic cell line that expresses a range of FcγRs, including both inhibitory and activating FcγRs making it a highly useful assay to measure ADP function in both HIV infection and vaccine-mediated protection

{Ackerman:2011eo}. We developed a modified assay, the ADP-SHIP assay, which is able to specifically discriminate between surface bound and internalized targets by flow cytometry without the need of employing imaging techniques. Although the standard ADP and the ADP-SHIP assay displayed comparable results, the dynamic range of responses detected was greater for the ADP-SHIP assay. Further, this assay can reliably detect the number of beads internalised per cell and the proportion of target cells with only surface-bound beads. It remains to be shown whether the ADP-SHIP assay offers any improvement in assessing the functional significance of ADP in HIV immunity over the standard ADP. Although we did not find a difference in levels of surface bound beads between HIV- and HIV+ samples, it is possible that future studies assessing cells at different time points after exposing opsonized beads to the target cells might reveal subtle differences. We also note that both the standard ADP and the ADP-SHIP assays result in a level of background association and internalization of gp140-coated beads in the absence of HIV Abs. Thus, both assays measure the antibody-dependent increase in phagocytosis (shown by comparing assays of HIV- compared to HIV+ IgG samples) rather than the total level of phagocytosis.

Using the ADP-SHIP assay we demonstrated that ADP-mediating Abs develop early during infection and that ADP responses increase over time in the absence of treatment. Whether this increase in ADP activity is due to the overall increase in ADP Ab titers or due to the overall maturation of the ADP response (specificity and avidity) remains to be elucidated. Ongoing studies from our own lab have also shown that ADP responses are reduced by antiretroviral treatment (data not shown).

Inducing protective Ab responses through vaccination is a goal of HIV vaccine studies. There is recent evidence on a potential role for vaccine-induced ADP in protecting macaques from SIV infection {Barouch:2013jv}. The only human HIV vaccine trial to date

to show some success is the RV144 trial and we therefore analysed plasma samples from a subset of vaccinees for ADP responses. We could not detect HIV Env-specific ADP responses to either B or A/E subtype Env proteins following vaccination. A conference presentation has suggested at least low-level of ADP may be induced by the RV144 regimen (add ref Chung, *Retrovirology* 2012, **9**(Suppl 2):P361). It will be important to analyse further samples from RV144 trial with more sensitive ADP assays in future studies. Although speculative, it is possible that improved immunity to HIV may be achievable if vaccine regimens induce a broader range of non-neutralising Ab responses, including ADP.

To conclude, the new ADP-SHIP assay now provides a valuable and specific tool to further study ADP-mediating antibodies in the context of vaccination and HIV infection.

Methods

Patients and plasma samples. To establish the new ADP-SHIP assay we studied serum samples from HIV-infected subjects (n=20) at various disease stages and from healthy subjects (n=20). Further longitudinal serum samples from patients (n=31) during primary and early chronic infection (4.7 months after infection and 25.9 months after infection) were also assessed for their ADP activity. The list of patients and their clinical data (VL, CD4 count, time point of infection) is presented in table 1. To investigate ADP after vaccination serum samples of randomly selected vaccine recipients (n=30) and placebo recipients (n= 26) of the RV144 vaccine trial {RerksNgarm:2009bk} were tested at week 26 of the trial. Informed consent was obtained from all subjects, and the study was approved by the respective institutional ethics committees.

HIV-IG (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog #3957 from NABI and NHLBI) was used as a positive control in all ADP assays.

IgG purification from plasma. Total IgG was purified from 50-100µl plasma using the Protein G HP Multitrap and the Antibody Buffer Kit (both GE Healthcare) according to manufacturer's instructions. Elution was repeated two times and pooled elutes were washed twice with PBS using 30k Amicon® Ultra-4 Centrifugal Filter Units (Millipore). IgG concentration was determined using the NanDrop 2000.

HIV Env gp140 protein and biotinylation. HIV Env gp140 protein (subtype B: AD8 clone of ADA; subtype A/E: 966, clone 93TH966-89) was secreted by stably transfected HeLa cells and purified by lentil-lectin affinity chromatography and size exclusion chromatography using a 16/60 Superdex 200 column as previously described {Center:2009dv}. Fractions enriched for gp140 trimers were pooled and concentrated. Briefly, 20-200 ug of gp140 Env was then biotinylated using EZ-Link® Sulfo-NHS-LC-Biotin kit (Thermo Scientific) according to the manufacture's instructions using a 20-fold biotin reagent excess. Excess biotin was removed by washing twice with PBS using the 30k Amicon® Ultra-4 Centrifugal Filter Units (Millipore).

Standard ADP assay. The standard ADP assay was performed as previously described {Ackerman:2011eo}. Briefly, 0.75µg of biontinylated gp140 (unless stated otherwise gp140 AD8 was used) and 3µl FITC-labeled NeutrAvidin® FluoSpheres® (beads 1µm) (Invitrogen) were incubated together overnight at 4 °C and subsequently washed with 2% BSA-PBS to remove unbound gp140. 10μl of 1:10 diluted coated beads were opsonised with 1µg/ml purified human IgG, for 2 h at 37 °C before incubated with 1x10e5 THP1 cells (monocytic cell line, ATCC→ TIB-202) in a total volume of 200μl. After an incubation of 16 h at 37 °C cells were washed with PBS fixed in 1% formaldehyde before acquired on a LSRII (BD) and analysed using FlowJo analysis software version 9.6.2.

ADP-SHIP assay. The ADP-SHIP assay utilises a specific hybridisation internalisation probe (SHIP). Similar to the standard ADP 0.75μg biotinylated HIV Env gp140 together

with 3μl of FITC-labelled NeutrAvidin® FluoSpheres® (beads 1µm) (Invitrogen) and 3µl of 150µM biotin- and Cy5-labeled florescent internalization probe (FIP_{Cy5}) (5' Cy5-TCAGTTCAGGACCCTCGGCT-N3 3', Integrated DNA Technologies, USA) {Liu:2013um} were incubated overnight at 4 °C. Beads were then washed twice with 2% BSA-PBS to remove unbound gp140 and FIP_{Cy5}. 10µl of a 1:10 dilution of coated beads were opsonised with 1µg/ml purified human IgG for 2 h at 37 °C, and then incubated with 1x10e5 THP1 cells in a total volume of 200μl. After a 16 h incubation at 37 °C cells were washed with ice cold PBS and surface bound beads were quenched by adding 1.01 μg/μl of the complementary quenching probe (QP_C) (5' AGCCGAGGGTCCTGAACTGA-BHQ2 3' Integrated DNA Technologies, USA) for 10 min at 4°C (Liu, 2013). Cells were fixed in 1% formaldehyde before acquired on the LSRII (BD) and analysed using FlowJo analysis software version 9.6.2. The proportion of cells with surface-bound or internalised beads was determined by flow cytometry as shown in Fig 2A. The number of beads internalized (1, 2 or >2) was determined by gating on the peaks associated with internalized beads as shown in Fig 3C (right panel). There was a high degree of concordance of this analysis when we assessed 10 replicates of a HIV+ sera samples, with the co-efficient of variation of 1, 2, and >2 beads per cell being 3.89%, 3.62%, and 3.56% respectively.

Deconvolution microscopy. Samples used for microscopy were treated the same way as described for the ADP and ADP-SHIP assay with the exception that a membrane staining step was performed before fixing. Briefly, 30µl of 5µg/ml of Wheat germ agglutinin (Alexa Fluor®594, Life Technologies) was added to the cells and incubated for 15 min at 4°C. Cells were subsequently washed with PBS and fixed in 30µl 4% formaldehyde before being imaged on an Applied Precision DeltaVision Deconvolution Microscope. Deconvolution and image processing was perfomed using SoftWorx software.

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References

Figure legends

Figure 1. Antibody-dependent phagocytosis (ADP) assays. (A) Standard ADP assay setup is shown as described in {Ackerman:2011eo}. FITC-labelled beads are coated with HIV envelope protein (Env) gp140 and incubated with HIV- or HIV+ purified IgG. (left panel). The opsonised beads are then incubated with the monocytic cell line THP1 and "phagocytosed" beads are identified by flow cytometry as FITC+ cells. This assay does not distinguish if beads are actually internalised or if they only bind to the surface of the cell (middle and right panel). (B) The new ADP-SHIP assay is shown. FITC-labeled beads are coated similar to the standard ADP assay, with HIV Env gp140 but also with a fluorescent internalisation probe (FIP_{Cy5}) and incubated with HIV- or HIV+ purified IgG (left panel). The opsonised beads are then incubated with the monocytic cell line THP1 and "phagocytosed" beads are measured by flow cytometry as FITC+ Cy5+ double positive cells (middle panel). The addition of a complementary quenching probe (QP_C) specifically blocks the Cy5 fluorescence derived from FIP_{Cy5} on surface bound beads, but not from internalised beads (right panel). Surface bound beads are FITC+ single-positive and internalised beads are double fluorescent (FITC+ Cy5+).

Figure 2: Validation of the new ADP-SHIP assay. A) Gating strategy for ADP-SHIP assay. The first 2 panels show gating strategy on live single cells. The third panel shows gating on bead+ cells (i.e. cells associated with FITC+ beads, either internalized or on the surface). The fourth panel shows gating on FITC+Cy5+ (internalized beads) and FITC+Cy5- (surface associated beads) cells. The filled histogram shows a 4°C negative control where minimal numbers of cells have internalized beads. The open histogram shows a sample incubated at 37 °C where 80.9% of cells associated with beads have at least one internalized bead. B) Equal numbers of bead+ cells for both the standard ADP

and the ADP-SHIP assay was detected. C) Deconvolution microscopy of ADP-mediated uptake. The left panel shows the standard ADP assay using FITC-labelled beads. The arrow for this panel shows beads associating with cells but does not discriminate whether the beads are surface bound or internalised. The right panel shows the ADP-SHIP assay. Arrows for this panel indicate either surface bound beads (FITC+Cy5-; green) or internalised beads (FITC+Cy5+; orange). The cell membrane was stained with wheat germ agglutinin Alexa Flour 594 (blue). Complementary quenching probe: QP_C

Figure 3. Comparison of antibody-dependent phagocytosis with the standard ADP and the ADP-SHIP assays. A) The phagocytic activity of 20 HIV-infected and 20 healthy individuals was measured using purified IgG from plasma. Readout is as follows: standard ADP assay: phagocytic score (% beads positive cells x MFI) / 10^4) (left y-axis); ADP-SHIP assay: % FITC+ and Cy5+ cells (right y-axis). The error bars represent mean ±SEM. **** p <0.0001 was determined using Student's T-test. B) Levels of surface bound beads in the ADP-SHIP assay are similar in 20 HIV+ and 20 HIV- IgG samples. Analysis of the number of beads per cell (internalized (FITC+ Cy5+) vs. surface bound (FITC+ Cy5-) beads) using the ADP-SHIP assay. In this assay, 29.7% of cells have at least one internalised bead (middle panel) and this can be broken down to cells with 1, 2 or >2 beads by gating on the peaks as shown in the right panel. D) Analysis of the numbers of internalised beads in the presence of 20 HIV-positive sera. The error bars represent mean ±SEM.

Figure 4: Development of ADP antibodies in HIV-infected patients during primary and early chronic infection. A) ADP responses were evaluated in HIV-infected individuals (n=31) using paired serum samples. Median primary infection was 4 months and median early chronic infection was 2 years 1 month. **** p <0.0001 was determined using Wilcoxon

Matched-Pairs Signed Rank test. B) Analysis of the number of beads per cell comparing internalised (FITC+Cy5+) and C) surface bound (FITC+Cy5-) beads in the presence of HIV Abs during primary and early chronic HIV infection (n=31). The error bars represent median ± IQR. **** $p<0.05$ was determined using Wilcoxon Matched-Pairs Signed Rank test. D) Correlation of ADP responses with months after HIV infection (n=31) $R^2= 0.55$ and $p <0.0001$ determined using Pearson's correlation.

Figure 5: Assessment of ADP in the RV144 HIV vaccine and placebo recipients. ADP was determined for 30 vaccine and 26 placebo recipients at week 26 of the trial. As a positive control ADP for 15 HIV-infected subjects was measured. The grey dotted line indicates ADP for an HIV-negative subject. ADP was measured against A) Env gp140 subtype B (strain AD8) and B) Env gp140 subtype A/E (strain 966). The error bars represent mean ± SEM. Surface-bound beads are shown for HIV-1_{AD8} and HIV-1_{966} gp140-coated beads in C) and D) respectively.

Figure S1: Efficient phagocytosis in both ADP assays is highly dependent on optimal HIV Env gp140 biotinylation and Env gp140 concentration and IgG concentration. (A) Comparing coating conditions of fluorescent beads were tested for both ADP assays. The beads were labelled using three different conditions (i) adding FIP_{cy5} and 0.75µg gp140 at the same time, (ii) adding FIP_{cy5} first before adding 1.5µg gp140 (gp140 in excess), and (iii) adding 1.5 µg gp140 (gp140 in excess) only as a control. patient 1 and patient 2: IgG from two HIV+ individuals; HIVIG: pooled HIV human immunoglobulin; HIV-: IgG from a pool of HIV- subjects; PBS: no IgG added. (B) Gating for internalised and surface bound beads was established performing the assay at 4˚C (no phagocytosis; binding only) and 37˚C (phagocytosis) using HIV+ and HIV- IgG. Q+: QP_C added; Q-: QP_C not added. (C) Optimal gp140 and IgG concentration was determined using the standard ADP and gp140

(shown here for subtype A/E (966)). Grey line represents background detected for non-opsonised beads (without IgG). Biotinylation of gp140 was performed with 10 to 100 fold molar excess biotin based on the manufacture's recommendations. (D) Optimal concentration of biotin for maximum biotinylation was determined by ELISA using streptavidin. (E) The degree of masking immunogenic epitopes on gp140 by attached biotin at varying levels of excess biotin was determined by using ELISA and pooled HIV+ serum. F) Levels of surface-associated beads in the presence and absence of gp140 coating of beads. Ten replicates were studied in the absence of sera and the co-efficient of variation noted on the graph. G) Reproducibility of ADP and ADP-SHIP assays. Ten replicates of a single HIV+ IgG sample were assessed by both assays and the co-efficient of variation shown for both assays.

Tables

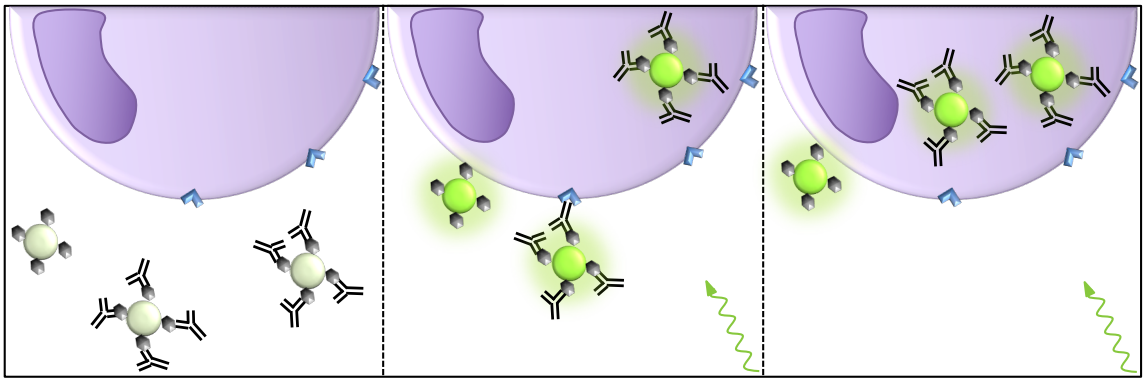
Table 1: Cohort characteristics for primary and early chronic HIV infection

	Primary Infection	Early Chronic Infection
Median CD4 count (Range)	723 cells/µl (168-1220)	570 cells/µl (76-818)
Median HIV-Viral Load (Range)	4.01 Log10 copies/ml plasma (2-5.88)	4.19 Log10 copies/ml plasma (1.69-5.32)
Median Time after Infection (Range)	4 months (1-11)	25 months (18-34)
Age (Range)	35 years old (25-62)	36.5 years old (28-64)

FIGURE 1

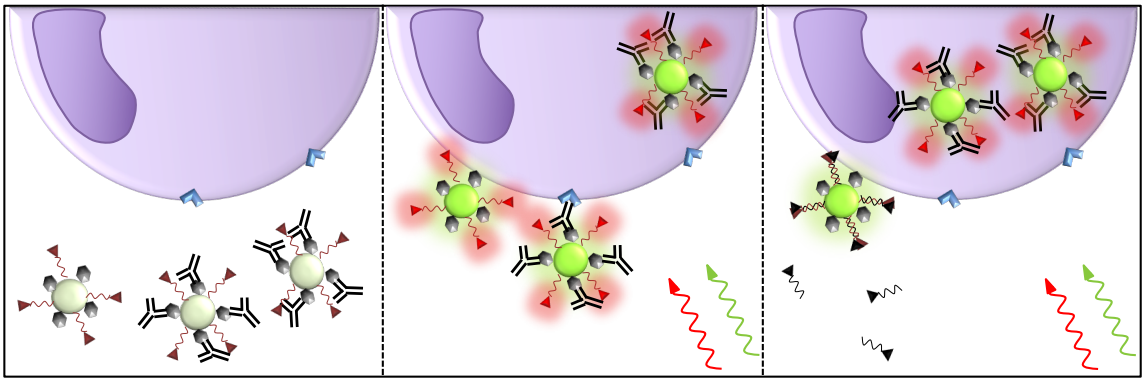
A

Standard Antibody-Dependent Phagocytosis Assay



B

Antibody-Dependent Phagocytosis SHIP Assay



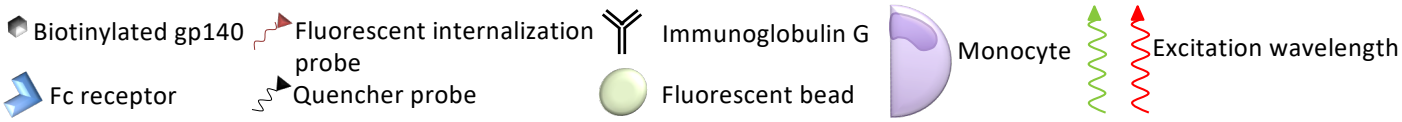


FIGURE 2

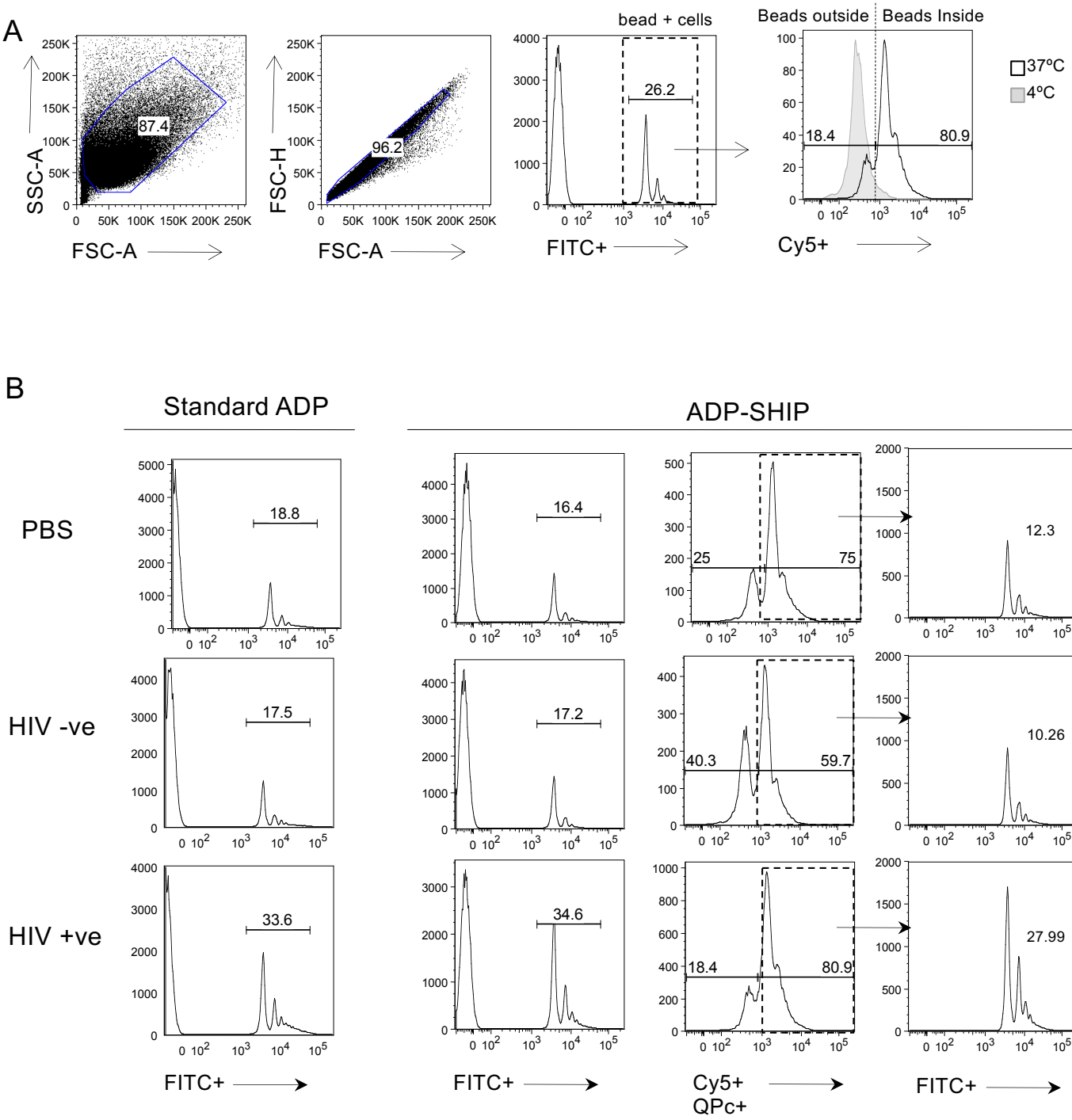

A
SSC-A
FSC-A
87.4
FSC-H
FSC-A
96.2
bead + cells
26.2
FITC+
Beads outside
Beads Inside
18.4
80.9
Cy5+
37ºC
4ºC
B
Standard ADP
ADP-SHIP
PBS
HIV -ve
HIV +ve
18.8
16.4
25
75
12.3
17.5
17.2
40.3
59.7
10.26
33.6
34.6
18.4
80.9
27.99
FITC+
FITC+
Cy5+
QPc+
FITC+
C
Standard ADP
ADP-SHIP

FIGURE S1

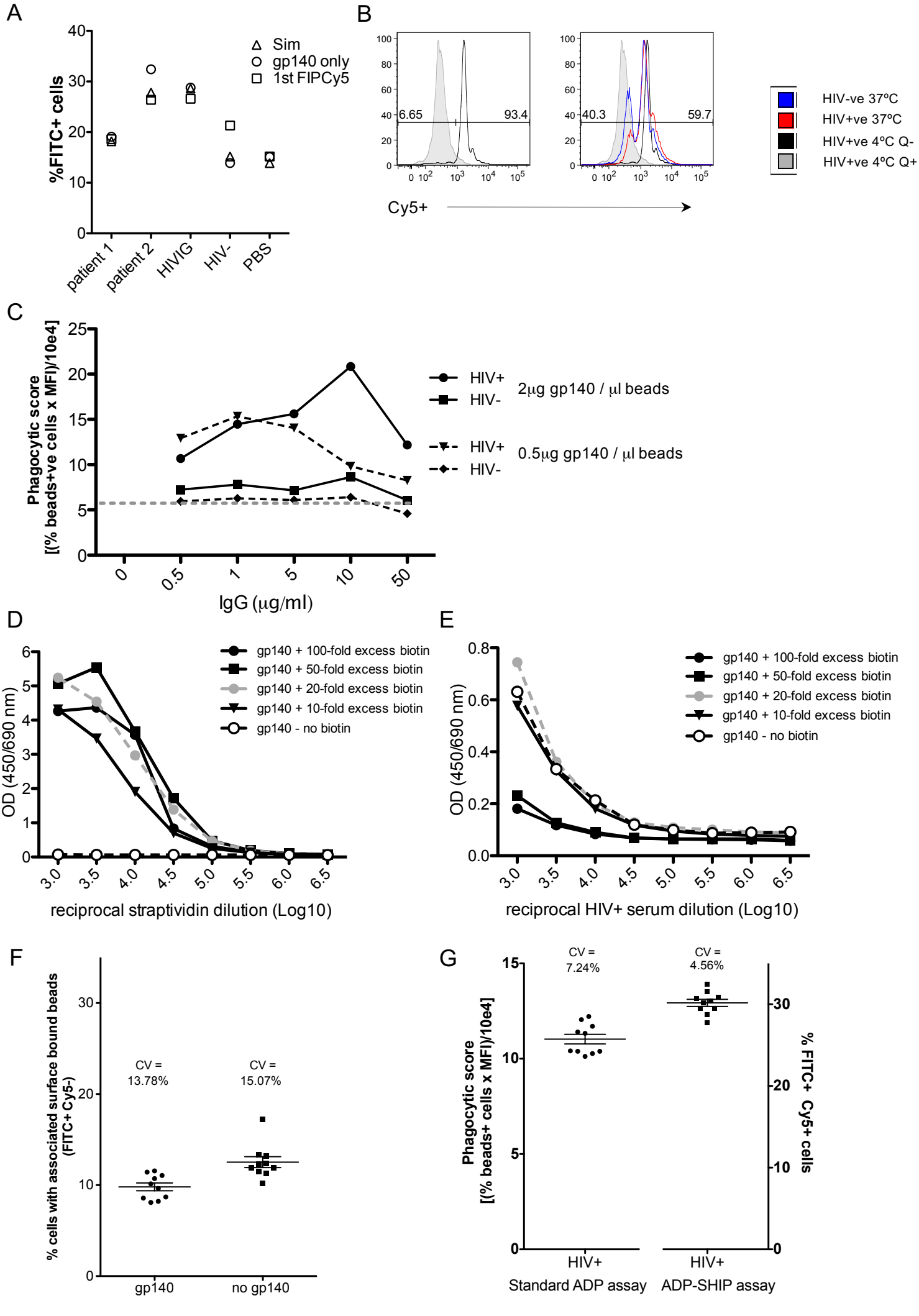

A
%FITC+ cells
Sim
gp140 only
1st FIPCy5
patient 1
patient 2
HIVIG
HIV-
PBS
B
6.65
93.4
40.3
59.7
Cy5+
HIV-ve 37ºC
HIV+ve 37ºC
HIV+ve 4ºC Q-
HIV+ve 4ºC Q+
C
Phagocytic score
[(% beads+ve cells x MFI)/10e4]
HIV+
HIV-
2µg gp140 / µl beads
0.5µg gp140 / µl beads
IgG (µg/ml)
D
OD (450/690 nm)
gp140 + 100-fold excess biotin
gp140 + 50-fold excess biotin
gp140 + 20-fold excess biotin
gp140 + 10-fold excess biotin
gp140 - no biotin
reciprocal straptividin dilution (Log10)
E
OD (450/690 nm)
reciprocal HIV+ serum dilution (Log10)
F
% cells with associated surface bound beads
(FITC+ Cy5-)
CV = 13.78%
CV = 15.07%
gp140
no gp140
G
Phagocytic score
[(% beads+ cells x MFI)/10e4]
% FITC+ Cy5+ cells
CV = 7.24%
CV = 4.56%
HIV+
HIV+
Standard ADP assay
ADP-SHIP assay

FIGURE 3

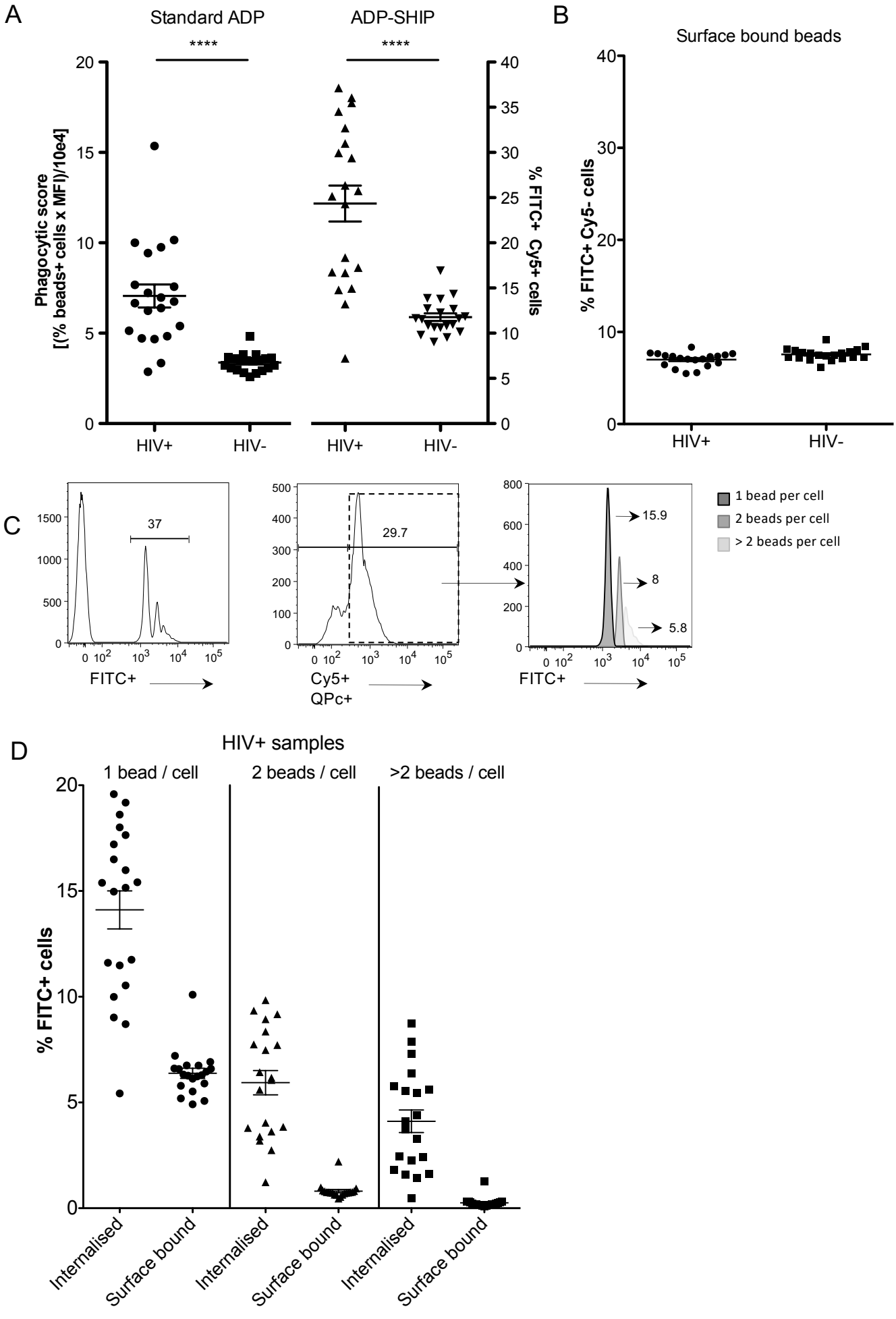

A
Standard ADP
ADP-SHIP
****
****
Phagocytic score
[(% beads+ cells x MFI)/10e4]
% FITC+ Cy5+ cells
HIV+
HIV-
HIV+
HIV-
B
Surface bound beads
% FITC+ Cy5- cells
HIV+
HIV-
C
37
29.7
15.9
8
5.8
FITC+
Cy5+
QPc+
FITC+
1 bead per cell
2 beads per cell
> 2 beads per cell
D
HIV+ samples
1 bead / cell
2 beads / cell
>2 beads / cell
% FITC+ cells
Internalised
Surface bound
Internalised
Surface bound
Internalised
Surface bound

FIGURE 4

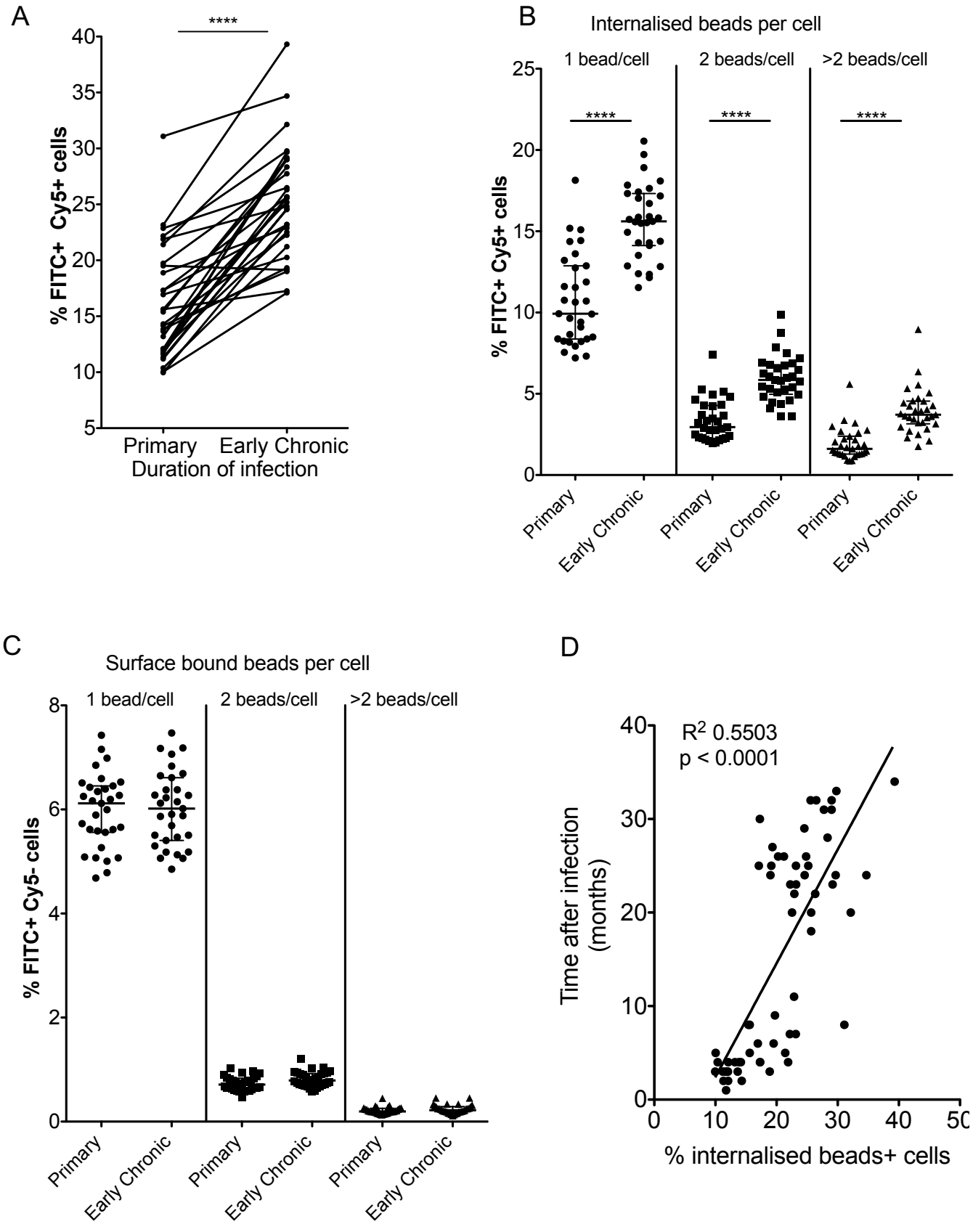
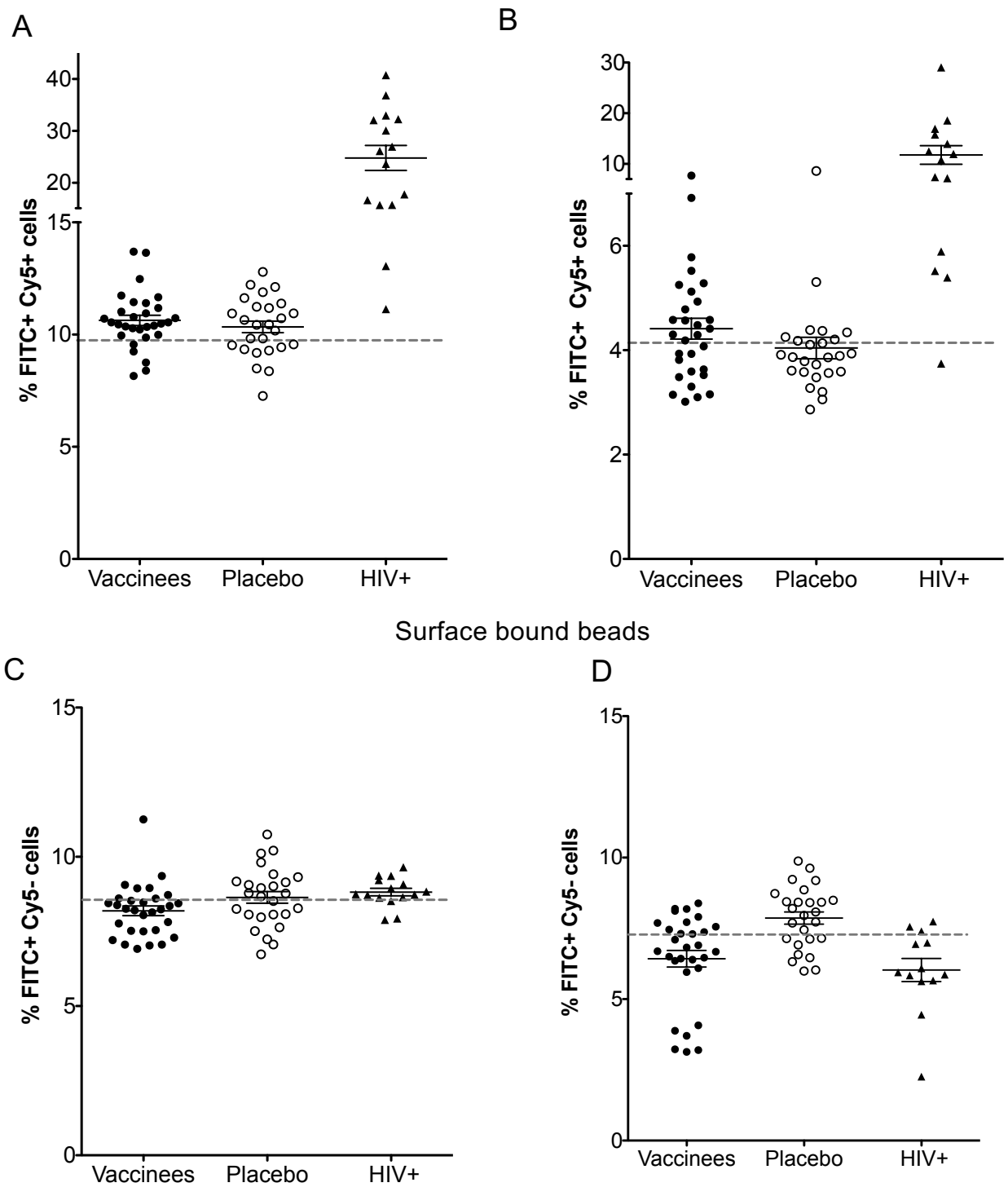

A
****
% FITC+ Cy5+ cells
Primary
Early Chronic
Duration of infection
B
Internalised beads per cell
1 bead/cell
2 beads/cell
>2 beads/cell
% FITC+ Cy5+ cells
Primary
Early Chronic
C
Surface bound beads per cell
1 bead/cell
2 beads/cell
>2 beads/cell
% FITC+ Cy5- cells
D
R2 0.5503
p < 0.0001
Time after infection (months)
% internalised beads+ cells

FIGURE 5


A
B
% FITC+ Cy5+ cells
Vaccinees
Placebo
HIV+
Surface bound beads
C
D
% FITC+ Cy5- cells