



Research Article

HIV-specific CD4-induced Antibodies Mediate Broad and Potent Antibody-dependent Cellular Cytotoxicity Activity and are Commonly Detected in Plasma from HIV-infected Humans



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ARTICLE INFO

Article history:

Received 3 August 2015

Received in revised form 31 August 2015

Accepted 1 September 2015

Available online 5 September 2015

Keywords:

ADCC

RF-ADCC

Antibody

HIV

CD4-induced

CD4i

C11

Potency

Breadth

ABSTRACT

HIV-specific antibodies (Abs) can reduce viral burden by blocking new rounds of infection or by destroying infected cells via activation of effector cells through Fc–FcR interaction. This latter process, referred to as antibody-dependent cellular cytotoxicity (ADCC), has been associated with viral control and improved clinical outcome following both HIV and SIV infections. Here we describe an HIV viral-like particle (VLP)-based sorting strategy that led to identification of HIV-specific memory B cells encoding Abs that mediate ADCC from a subtype A-infected Kenyan woman at 914 days post-infection. Using this strategy, 12 HIV-envelope-specific monoclonal antibodies (mAbs) were isolated and three mediated potent ADCC activity when compared to well-characterized ADCC mAbs. The ADCC-mediating Abs also mediated antibody-dependent cell-mediated virus inhibition (ADCVI), which provides a net measure of Fc receptor-triggered effects against replicating virus. Two of the three ADCC-mediating Abs targeted a CD4-induced (CD4i) epitope also bound by the mAb C11; the third antibody targeted the N-terminus of V3. Both CD4i Abs identified here demonstrated strong cross-clade breadth with activity against 10 of 11 envelopes tested, including those from clades A, B, C, A/D and C/D, whereas the V3-specific antibody showed more limited breadth. Variants of these CD4i, C11-like mAbs engineered to interrupt binding to FcγRs inhibited a measurable percentage of the donor's ADCC activity starting as early as 189 days post-infection. C11-like antibodies also accounted for between 18–78% of ADCC activity in 9 chronically infected individuals from the same cohort study. Further, the two CD4i Abs originated from unique B cells, suggesting that antibodies targeting this epitope can be commonly produced. Taken together, these data provide strong evidence that CD4i, C11-like antibodies develop within the first 6 months of infection and they can arise from unique B-cell lineages in the same individual. Further, these mAbs mediate potent plasma IgG-specific ADCC breadth and potency and contribute to ADCC activity in other HIV-infected individuals.

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1. Introduction

Antibodies (Abs) can prevent viral infection through two key mechanisms: neutralizing cell-free virus and targeting virally-infected cells for destruction through either antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP) (reviewed in Ackerman and Alter, 2013). While considerable effort has been placed on characterizing broadly neutralizing antibodies (bnAbs) due to their protective capacity in non-human primates, far less is known about ADCC-mediating Abs (reviewed in

Ackerman and Alter, 2013; Mascola and Montefiori, 2010; Burke and Barnett, 2007; West et al., 2014).

Abs that facilitate ADCC or antibody-dependent cell-mediated viral inhibition (ADCVI) activity, a related metric that quantifies multiple Ab-specific effects on viral replication including ADCC, develop in most infected individuals within the first year following infection and facilitate activity through effector cell mechanisms (Forthal et al., 2001a; Chung et al., 2011a; Alpert et al., 2012; Dugast et al., 2014a). Landmark experiments conducted by Hessel et al. effectively demonstrated a role for antibodies mediating Fc receptor (FcR)-driven effector function by proving that administration of a variant of monoclonal antibody (mAb) b12 that was unable to engage FcRs was 50% less protective against both a high- and low-dose SHIV challenge than mAb b12 with

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complete effector function, suggesting that both Ab ADCC and neutralizing activity contribute to protection (Hessell et al., 2007, 2009a). Indeed, multiple studies conducted in HIV-infected populations (reviewed in Lewis, 2014), SIV-infected macaques (Banks et al., 2002; Asmal et al., 2011; Sun et al., 2011) and HIV-infected humanized mice (Bournazou et al., 2014) have similarly established a relationship between HIV-specific ADCC or ADCVI antibodies and disease pathogenesis, including reduced viral load (Forthal et al., 2001a; Baum et al., 1996; Ahmad et al., 2001; Nag et al., 2004; Lambotte et al., 2009, 2013; Johansson et al., 2011; Wren et al., 2013), increased CD4 T cell counts (Ahmad et al., 2001, 1994; Chung et al., 2011b) and slower disease progression (Forthal et al., 2001a; Baum et al., 1996; Ahmad et al., 2001; Chung et al., 2011b). These findings suggest that early ADCC responses generated against a new infection may help control virus levels following acquisition. Moreover, Milligan et al. (2015) demonstrated that increased levels of passively acquired HIV-specific ADCC antibody activity in infants correlate with survival, providing evidence for a protective effect of pre-existing antibodies on clinical outcome (Milligan et al., 2015). There is also evidence that ADCC antibodies may impact transmission. Mabuka et al. (2012) correlated higher breast milk IgG ADCC activity that was independent of neutralizing activity with a reduced risk of vertical transmission between mother–infant pairs, providing evidence for a potentially unique role for ADCC in the index case in reducing transmission risk (Mabuka et al., 2012). However, other studies have not detected a significant effect on protection with ADCC-mediating mAbs in either human cohort or macaque model studies (Florese et al., 2006; Dugast et al., 2014b), and it is unclear whether these observations reflect differences in the protective efficacy of the antibodies tested or other methodological factors.

Vaccine studies conducted in both non-human primate models (Alpert et al., 2012; Gómez-Román et al., 2005; DeVico et al., 2007; Hidajat et al., 2009; Xiao et al., 2010; Brocca-Cofano et al., 2011; Barouch et al., 2012) and humans (Forthal et al., 2007; Haynes et al., 2012) have also suggested that ADCC activity may provide some protection against infection. Early evidence from the Vax004 Phase III efficacy study identified an inverse correlation between ADCVI activity and infection (Forthal et al., 2007). Most recently, an immune-correlates analysis of the RV144 vaccine trial showed a correlation between higher ADCC activity and protection from infection among vaccinees with low plasma IgA, lending additional support for the potential of ADCC-mediating antibodies to protect (Haynes et al., 2012; Tomaras et al., 2013).

ADCC-mediating antibodies target a wide range of epitopes on the envelope (Env) protein, as well as epitopes on Gag (Grunow et al., 1995), Pol (Isitman et al., 2012), Nef (Yamada et al., 2004) and Vpu (Wren et al., 2013; Tiemessen et al., 2009) proteins, though the significance of ADCC activity directed against proteins other than Env remains unclear. Within Env, ADCC-mediating antibodies target epitopes similar to neutralizing antibodies, including the membrane proximal external region (MPER), CD4 binding site (CD4bs) and V1-V2 and V3 regions as well as unique epitopes on the gp120 inner domain naturally occluded by gp41 and exposed following CD4 binding (CD4-induced [CD4i] epitopes) (reviewed in (Pollara et al., 2013; Gach et al., 2011)). A detailed study by Guan et al. (2013) finely mapped three unique clusters (A, B and C) of CD4i-specific antibodies using a competition-based ELISA approach (Guan et al., 2013). Cluster A epitopes become exposed following the conformational change resulting from Env binding to cellular CD4 that occurs during viral entry or following infection and subsequent cell–cell spread when CD4 and the viral envelope are co-expressed on the surface of the same cell (Finnegan et al., 2001; Acharya et al., 2014; Veillette et al., 2014). Prototypic Cluster A mAbs A32 and C11 bind to similar epitopes on gp120 but only weakly cross-compete with each other, suggesting they have unique specificities (Guan et al., 2013). Fab inhibition experiments have demonstrated that A32-like antibodies frequently constitute the majority of the ADCC activity observed in chronically-infected (Ferrari et al., 2011) and RV144-vaccinated (Bonsignori et al., 2012) individuals. The Cluster B

epitope is defined by only one antibody, N12-i15, and targets a conformational epitope on gp120 that requires CD4 binding and involves the V1–V2 loop. Cluster C antibodies recognizing the co-receptor binding sites (CoRBS) are the only CD4i antibodies capable of neutralizing cell-free virus and are further sub-categorized into 4 groups as specified by competition with either 17b or 19e mAbs. Among these ADCC-mediating antibodies, the A32 and C11 mAbs from cluster A are the most potent (Guan et al., 2013), and these CD4i mAbs have non-overlapping specificities. Few studies have examined ADCC mAb breadth or the ability to recognize diverse envelopes from different HIV-1 clades.

Recently, two studies identified epitope breadth (Wren et al., 2013) and increased cross-clade ADCC activity (Madhavi et al., 2014) as characteristics associated with superior ADCC-mediating antibody responses. Despite this work, we still know very little about the features defining a protective ADCC-mediating immune response or the optimal methods required to dissect this response at the monoclonal level. Further, few studies have attempted to define the contribution of epitope-specific mAbs other than A32 to the overall ADCC response or to determine the kinetics of ADCC Ab development following infection. While considerable progress in identifying HIV-specific bnAbs has been made using high-throughput methods such as protein-specific screens and microneutralization assays, no high-throughput, functional methods for isolating ADCC-mediating antibodies have been described.

In this report, we screened peripheral blood mononuclear cells (PBMCs) approximately 2.5 years post infection (PI) from an individual who demonstrated cross-clade ADCC and neutralizing Ab responses (Piantadosi et al., 2009; Bosch et al., 2010). Memory B cells that expressed B cell receptors (BCRs) that bound HIV envelope protein were isolated using HIV virus-like particles (VLPs). Using this approach, we identified three ADCC-mediating antibodies with cross-clade activity that targeted two distinct epitopes: the C11-like epitope, and the V3 region.

2. Methods

2.1. Ethics Statement

The Fred Hutchinson Cancer Research Center's, University of Washington's and University of Nairobi's Institutional Review Boards approved this study. Study participants provided written consent prior to enrollment.

2.2. Human PBMC and Plasma Samples

Study participants were enrolled as HIV naïve into a prospective cohort of HIV-1 negative high-risk women in Mombasa, Kenya and monitored by HIV serology and RNA testing to define the time of infection (Lavreys et al., 2002). Peripheral blood mononuclear cells (PBMCs) and plasma samples were obtained at regular intervals following seroconversion. All study participants were treated according to Kenyan National Guidelines and did not receive ARVs at any point during the period in which samples were analyzed for this study. QA255 seroconverted after 4.8 years of follow-up and had a setpoint viral load of 4.8 log copies/mL. Peripheral blood mononuclear cells (PBMCs) were obtained at regular intervals, including 914 days post HIV-infection when her CD4 count was 368 cells/mm³. IgG was purified from plasma samples using Melon Gel (Pierce), buffer-exchanged following manufacturer protocol (Zeba filter, Pierce) and quantified using Nanodrop. IgG was purified from 6 plasma samples obtained from QA255 between 89 and 914 days post-infection (dpi) and from 10 plasma samples obtained from cohort participants at a single time point 3 years PI. These additional 10 women were randomly selected from a subset of seventy women whose Nab breadth was characterized in a previous study (Piantadosi et al., 2009).

2.3. Monoclonal Antibodies and Peptide Reagents

The Consensus A peptide library was obtained from the AIDS Research and Reference Reagent Program (NIAID, NIH). Ab C11 was kindly provided by J. Robinson (Tulane University), Abs 17b and A32 by B. Haynes (Duke University) as well as the AIDS Research and Reference Reagent Program (NIAID, NIH) and the influenza-specific negative control Fl6v3 by J. Bloom (Fred Hutchinson Cancer Research Center). Ab A32 originated from a Clade B-infected individual (Moore et al., 1994a); the donor subtype from which Ab C11 originated has not been described (Moore et al., 1994b). MAbs b12, b6, PG9, and Den3 were kindly provided by D. Burton (TSRI). MAbs 2G12 and 1F7 were donated by Dietmar Katinger (Polymun Scientific) and mAbs VRC01 and VRC03 were kindly provided by J. Mascola. Q461.d1 linear peptides were provided by N. Sather and L. Stamatatos (Seattle Biomed).

2.4. Viral Like Particle Production

Viral like particles that include GFP fused to Vpr and expressing Q461.d1 Env and QA255.21P Env were generated in a similar manner as that described in (Hicar et al., 2010). Briefly, T-Rex 293 cells were transfected with plasmid pcDNA4/TO Gag (Zeocin resistance) and pcDNA5/TO encoding either QA255.21P or Q461.d1 Env (puromycin resistance) under a tetracycline-controlled cytomegalovirus promoter and then selected in Zeocin and puromycin to generate stable, doxycycline-inducible cell lines. Cell clones that exhibited optimal Gag and Env expression and complete cleavage of gp160 to gp120 and gp41 were selected, and subsequently transfected with pcDNA5-TO Vpr-GFP plasmid (hygromycin resistance) to generate Vpr-GFP-expressing cells. GFP-labeled VLPs were harvested after three days of doxycycline induction, centrifuged at low speed, filtered and pelleted via ultracentrifugation through a 20% sucrose cushion and purified on a second linear 20–60% sucrose gradient.

2.5. Sorting of Memory B Cells and Reconstruction of Antibodies

PBMCs were obtained 914 days post HIV-infection from study participant QA255. Approximately 6 million cryopreserved PBMCs were thawed in a 37 °C water bath and re-suspended in 5 mL of pre-warmed RPMI 1640 medium (Invitrogen) containing 10% FBS, followed by centrifugation at 300 × g for 10 min. CD19+ B cells were first enriched by negative depletion using magnetic particles coated with antibody complexes recognizing CD2, CD3, CD14, CD16, CD36, CD43, CD56, CD66b, glycoporin A and dextran according to the manufacturer's instructions (StemCell) before they were stained on ice for 30 min using a cocktail of anti-CD19-PE-Cy7 (BD Bioscience), anti-CD27-APC (BD Bioscience), and 50 µL of a 1:1 mixture of concentrated QA255.21p.A17 and Q461d1 GFP-VLPs. In total, 192 memory B cells (CD19+, CD27+) that bound to VLPs were sorted based on GFP expression directly into 96-well PCR plates containing 20 µL of lysis buffer/well (0.5 µL RNase Out (Invitrogen), 5 µL of 5× first strand buffer (Invitrogen), 1.25 µL of 0.1 M DTT (Invitrogen) and 0.0625 µL of Igepal (Sigma)) at a density of 1 cell/well. Plates were frozen immediately on dry-ice before storage at –80 °C.

The variable region of the heavy chain and the light chain immunoglobulin genes were amplified by RT-PCR as previously described (Tiller et al., 2008; Scheid et al., 2011). Briefly, cDNA was generated using Superscript III with random hexamers (Invitrogen), which provided templates for five independent PCRs. Three independent, semi-nested PCRs amplified the Igγ variable genes and two independent nested PCRs amplified Igκ and Igλ variable genes. PCRs for the heavy chain were performed as described by (Scheid et al., 2011) and (Wu et al., 2011) and for the light chain as described by (Tiller et al., 2008). Primer sets used to amplify the heavy chain variable regions were reported in (Georgiev et al., 2013) and the light chain variable regions were specified in (Tiller et al., 2008). Products from positive PCRs were sequenced

before cloning into the corresponding Igγ1, Igκ and Igλ expression vectors (kindly provided by Michel Nussenzweig). Cloned inserts were verified by sequencing. Final sequences were characterized by gene family, percent mutation from germline and CDR3 length using the IMGT database (www.imgt.org). LALA variants were generated by overlap extension PCR to introduce the L234A and L235A mutation into the Igγ1 expression vectors (Hezareh et al., 2001; Shields et al., 2001).

Paired heavy and light chain plasmids cloned from the same well were combined in all possible pairings if there was more than one heavy or light chain isolated. Paired heavy and light chain clones were co-transfected in equal ratios into 293F cells (1×10^6 cells/1 µg of total DNA) with a 4:1 293Fectin: DNA ratio. Antibodies were harvested 72 h following transfection, and IgG was purified using Protein G resin in hand-packed, gravity flow columns (Pierce) or Protein G spin columns (Pierce). Antibody concentration was determined using either a total IgG ELISA or protein absorbance at 280 nM (Nanodrop).

2.6. VLP, gp120 and Peptide ELISAs

2.6.1. VLP ELISA

VLP ELISA was adapted from (Hicar et al., 2010). In brief, Immunolon 2-HB plates were coated with 100 µL of VLPs diluted to 1×10^8 particles/mL in 0.1 M sodium bicarbonate coating buffer (pH 7.4) overnight at 4 °C. Plates were thoroughly washed with PBS-0.05% Tween wash buffer and blocked with 10% non-fat dry milk (NFDm) diluted into wash buffer. After at least 1 h, the milk block was removed, and 100 µL primary antibody diluted into the NFDm added for 1 h at 37 °C. The plates were thoroughly washed a second time, and 100 µL anti-IgG-HRP (Sigma, 1:2500 dilution) diluted in NFDm added for one hour incubation at room temperature. The plates were washed a third time, and 50 µL Ultra-TMB (Thermo Scientific) substrate was added for 10 min. The reaction was stopped by adding an equal volume of 0.1 M H₂SO₄ and the absorbance read within 30 min using 450 nM optical density. The endpoint titer was defined as the average Ab concentration with binding 2-fold greater than Influenza-specific Ab Fl6v3 negative control.

2.6.2. Gp120 and Peptide ELISAs

The gp120 and peptide ELISAs were performed as described above, where the only modification was the type and amount of bound antigen: gp120 protein was used at a final concentration of 0.5 µg/mL (50 ng coated per well), and the linear peptides were used at a final concentration of 2 µg/mL (200 ng coated per well). The limit of detection (LOD) was defined as two times the binding measured with Influenza-specific Ab Fl6v3.

2.6.3. Competition ELISAs

For competition binding assays, the competitor antibody was serially diluted 4-fold at a starting concentration of 20 µg/mL and added to gp120-coated plates for 15 min before addition of 1.5 µg/mL biotinylated (BT)-C11 or 0.625 µg/mL BT-A32, both pre-defined limiting concentrations for an additional 45 min. Plates were washed in between each step and before addition of Ultra TMB-substrate and 0.1 M H₂SO₄ as described above. Relative BT-C11 binding was calculated by dividing BT-C11 binding in the presence of each competitor Ab by the average BT-C11 binding in the presence of Influenza Ab Fl6v3.

2.7. TZM-bl Neutralization Assay

Neutralization assays were performed as previously described (Goo et al., 2012). Reported IC₅₀ values are the average of two or three independent experiments performed in duplicate.

2.8. Fab Fragment Preparation

Fab fragments were generated following protocol instructions using the papain digestion kit (Pierce). In brief, 125 µg of IgG1 antibody was buffer exchanged into digestion buffer, and incubated with papain-coated agarose beads at 37 °C on a nutator for 5–7 h. The antibody was removed from the coated papain-coated beads and the F(ab) fragment was separated from Fc or undigested antibody using a Protein G column (Pierce) and buffer-exchanged (Zeba filter, Pierce).

2.9. Rapid and Fluorometric ADCC Assay (RF-ADCC)

The RF-ADCC assay was performed as described (Mabuka et al., 2012; Gómez-Román et al., 2006). In brief, CEM.NKr cells (AIDS Research and Reference Reagent Program, NIAID, NIH) were double labeled with a PKH26-cell membrane dye (Sigma Aldrich) and a cytoplasmic-staining CFSE dye (Vybrant CFDA SE Cell Tracer Kit, Life Technologies) and coated with gp120 protein (Immune Tech) for 1 h at room temperature at a ratio of 1.5 µg of protein (1 µg/µL) per 1×10^5 double-stained target cells. Coated target cells were washed once with complete RPMI media (Gibco) supplemented with 10% FBS (Gibco), 4.0 mM Glutamax (Gibco) and 1% antibiotic-antimycotic (Life Technologies). Monoclonal antibodies or plasma samples were diluted in complete RPMI media and mixed with 5×10^3 target cells for 10 min at room temperature before PBMCs obtained from an HIV-negative donor were added at a ratio of 50 effector cells: 1 target cell. Coated target cells, antibody dilutions and effector cells were co-cultured for 4 h at 37 °C before being fixed in 150 µL 1% paraformaldehyde (Affymetrix). Eighty percent (80%) of the cell volume was acquired by flow cytometry (LSR II, BD) and ADCC activity was defined as the percent of PE+, FITC-cells where background (Ab-mediated killing of uncoated cells) was between 3–5% as analyzed using FlowJo software (Treestar).

Relative ADCC activity was calculated by dividing the percent ADCC activity at each dilution minus background by the maximum percent ADCC activity measured across all dilutions. The data were plotted with relative ADCC activity on the y-axis and \log_{10} antibody concentration on the x-axis. The effective concentration 50 (EC₅₀) was determined by fitting a sigmoidal dose–response curve to the data and identifying the concentration responsible for 50% ADCC activity (Graphpad Prism v6.0f).

2.9.1. Cross-clade Analysis

In the cross-clade ADCC assays, all antibodies including QA255 914 plasma IgG were tested at a single concentration. This concentration was chosen based on experiments that defined peak ADCC activity against Q461.d1-coated cells using a range of dilutions of the IgG. Percent ADCC activity was reported if activity was 2-fold greater than that measured in the presence of an influenza-specific Ab (Fl6v3) after subtracting away background (Ab-mediated killing of uncoated cells). Antibodies that did not mediate activity against specific gp120s were indicated to have no activity (NA).

2.9.2. Fab Blocking

In the Fab blocking experiments, gp120-coated target cells were incubated with 25 µg/mL A32 or 17b Fab or 50 µg/mL C11 Fab for 10 min at room temperature prior to addition of full-length antibody diluted to a concentration responsible for peak ADCC activity as described above. Gp120-coated cells incubated with A32 or 17b Fab fragments were washed once prior to addition of the competitor antibody and the assay completed as described above. In order to maintain sufficient reduction of autologous ADCC activity, C11 Fab was not washed away prior to addition of full-length antibody. Relative ADCC activity is defined as Ab-specific ADCC activity minus background in the presence of each Fab as compared to Ab-specific ADCC activity in the absence of

the Fab. Average ADCC activity for each Ab in the absence of Fab was set to 1.0.

2.9.3. LALA Inhibition

In order to ensure complete inhibition of autologous mAb controls, the Q461.d1 gp120 protein was diluted to 37.5 ng/µL before coating CEM cells. As in the C11 Fab experiments, gp120-coated CEMs were incubated with 25 µg/mL LALA antibodies for 10 min prior to addition of full-length, wildtype effector antibodies. Plasma IgG was mixed with coated, double-stained target cells at 2.5 µg/mL. Relative ADCC activity is defined as autologous Ab, QA255.914 IgG or A32 activity minus background in the presence of the specified LALA variant as compared to Ab-specific ADCC activity in the absence of the LALA variant.

2.10. Antibody-dependent Cellular Viral Inhibition (ADCVI) Assay

1×10^7 CEM.Nkr.CCR5 cells were spin-infected with Q461.d1 virus at an MOI of 1 in the presence of 10 µg/mL polybrene at room temperature for 2 h at 2300 rpm. Cells were then re-suspended in additional RPMI medium plus 10 µg/mL polybrene and cultured for 72 h at 37 °C. After cell-free virus was removed by three subsequent washing steps 5×10^4 cells were mixed with HIV-specific Abs in triplicate 96-U bottom wells at a final concentration of 50 µg/mL in conjunction with effector cells (PBMCs) at an effector: target cell ratio of 10:1. The mixture of infected cells, antibody and effector cells were incubated together for 7 days. Virus production in the supernatant was subsequently measured by p24 ELISA (Zeptometrix) according to the manufacturers protocol. Percent ADCVI activity is expressed as the reduction in virus production compared to a non-specific Ab control (Den3). Data are the average of two independent experiments.

2.11. Graphics

Figures were prepared using Graphpad Prism (v6.0f).

3. Results

3.1. Isolation of Memory B Cells That Bound VLPs and Amplification of HIV-specific Abs

We used HIV VLPs labeled with GFP to isolate HIV-specific memory B cells from a study participant, QA255, who developed early cross-clade neutralizing activity (Piantadosi et al., 2009; Bosch et al., 2010). We used an equal mixture of two different VLPs to maximize our chances of capturing both autologous and cross-reactive mAbs. VLPs generated in this manner present primarily cleaved trimeric Env (Hicar et al., 2010). One VLP population expressed the envelope from Q461.d1, a clade A Kenyan strain that is recognized by QA255's contemporaneous plasma, as well as HIV-specific mAbs directed against the CD4 binding site, V1/V2 regions of gp120 protein and the MPER (Goo et al., 2012; Blish et al., 2008). The second VLP expressed QA255.21P, an Env identified from the individual under investigation at 21 days post-infection. One hundred and ninety-two VLP-binding memory B cells were obtained from approximately 129,000 CD19+, CD27+ B cells isolated from QA255 PBMCs at 914 dpi and sorted into individual wells of a 96-well plate.

Either a heavy or light chain variable region was amplified from 59% of wells and both were amplified from 30% of wells. Multiple unique heavy or unique light chain variable regions were amplified in 3 wells and in one of these cases two unique heavy and two unique light chain variable regions were amplified. Thus, all combinations of the variable heavy and light chains were tested from these three wells. Heavy and light chain pairings resulted in 48 antibodies from 43 distinct wells that expressed detectable IgG by ELISA (data not shown).

One of the two VLPs used to sort B cells, Q461.d1 VLP, was used to examine the antibodies for their ability to bind HIV Env protein. All antibodies were screened for binding against both enveloped and non-enveloped particles to discriminate HIV envelope-specific binding from non-specific VLP binding. Twelve of the 48 (25%) antibodies bound uniquely to the Q461.d1 VLP with endpoint titers (EPT) ranging from 24 ng/mL to 10,000 ng/mL, the highest concentration tested (Fig. 1A & B), and were thus considered to be HIV Env-specific.

Eight antibodies (17%) bound to both the enveloped and non-enveloped VLP (Fig. 1A) with EPTs ranging from 0.61 to 10,000 ng/mL and 0.70 to 7083 ng/mL for enveloped and non-enveloped VLPs, respectively (Table S1). Further analysis of these eight antibodies indicated that all of them bound to p24 protein as did anti-p24 specific Ab 71-31, which also bound equally well to the enveloped and non-enveloped VLP (Table S1). In contrast, two control antibodies that were Env-specific (QA255.157 and QA255.105) did not bind to either the p24 protein, or to the non-enveloped VLP (Table S1). Taken together, these data suggest that this second subset of Abs ($n = 8$, 17%) were HIV gag-specific.

No binding to either form of the Q461.d1 VLPs was detected with 28 of the 48 antibodies (58%; Fig. 1A), suggesting that they likely originated either from B cells that were strain-specific and bound only to QA255 VLPs (binding not tested) or were not HIV-specific, but were still captured in the sorting process. A third explanation is that these non-HIV specific Abs could have resulted from pairing of V_H and V_L chains that were amplified from two unique B cells captured in the same well that failed to reconstruct an HIV-specific mAb.

3.2. Three Antibodies Demonstrated Modest Neutralization Activity

All 48 antibodies were screened for neutralization activity against Q461.d1. Among the 48 Abs tested, three neutralized Q461.d1 with IC_{50} values ranging from 1.4 to 15.7 μ g/mL (Table S2). Two of the three antibodies, QA255.087 and QA255.187, demonstrated modest cross-clade neutralization (IC_{50} 1.6–10.8 μ g/mL) against a second neutralization-sensitive clade B variant SF162 (Table S2). However, none of the three antibodies neutralized any of the 7 Tier 2 viruses at the highest concentration tested (20 μ g/mL) (Table S2).

3.3. Three Antibodies Demonstrated Potent ADCC Activity

We next screened the antibodies for ADCC activity using the Rapid and Fluorometric ADCC (RF-ADCC) assay (Mabuka et al., 2012; Gómez-Román et al., 2006). The lysis of gp120-coated target cells measured by this assay reflects both the destructive activity of natural

killer cells as well as phagocytic activity of monocytes (Kramski et al., 2012) and has been associated with clinical outcome in HIV infections (Milligan et al., 2015). Since all of the variable heavy chain genes were expressed in an IgG1 vector, an effector portion that is capable of ADCC, any differences we observed in ADCC-activity would be due to the binding interaction with the Ab V_H & V_L regions. Three antibodies, QA255.105, QA255.157 and QA255.253, demonstrated ADCC activity against Q461.d1 Env at least 2-fold higher than background mediated by an influenza-specific monoclonal control, Fl6v3 (Corti et al., 2011) (data not shown). All three Abs were HIV envelope-specific based on VLP binding results (Fig. 1B). Antibody QA255.105 bound the Q461.d1 VLPs with an average EPT of 79 ng/mL, while QA255.157 and QA255.253 demonstrated more modest binding, with EPTs of 4170 ng/mL and 2500 ng/mL, respectively (Fig. 1B). QA255.105 also demonstrated modest neutralizing activity (Table S2). Examination of the endogenous IgG constant region sequence indicated that QA255.157 and QA255.253 were originally encoded as an IgG1 isotype (Table 1). Sequence data was unavailable to identify the original isotype of QA255.105.

To determine the potency of these three antibodies, we next quantified both the concentration of antibody required for 50% ADCC activity (50% effective concentration, or EC_{50}) and maximum percent killing and compared these data to potent, well-characterized ADCC-mediating Abs A32 (Moore et al., 1994a) and C11 (Moore et al., 1994b). The average EC_{50} values of the three QA255 Abs ranged from 6.3 to 11.2 μ g/mL, which were within 2–5 fold of A32 and C11 (average EC_{50} 2.2 and 3.3 μ g/mL, respectively; Fig. 2A, B). All five antibodies demonstrated similar maximum ADCC activity (Fig. 2C).

3.4. Antibodies That Demonstrated ADCC Also Mediated ADCVI Activity

While the RFADCC assay is of high relevance because its activity is associated with improved outcomes in HIV, it mainly measures activity to monomeric gp120, and not to the trimer found on virions and infected cells. Therefore, to determine whether the mAbs identified in the RF-ADCC assay also target envelope in its native state on infected cells, we next tested the Abs in the ADCVI assay, which uses replication-competent HIV (Forthal et al., 2007, 2001b). The activity in this assay has also been linked with reduced viral levels (Hidajat et al., 2009; Xiao et al., 2010; Brocca-Cofano et al., 2011; Florese et al., 2009), suggesting it too measures a biologically relevant antibody activity. We compared Abs QA255.157 and QA255.105 to a panel of nine mAbs, some of which have previously been reported to mediate ADCVI, including (i) CD4bs mAbs b12 (Hessell et al., 2007; Rosenberg et al., 2013), b6 (Pantophlet et al., 2003), VRC01 (Rosenberg et al., 2013; Wu et al., 2010), VRC03 (Wu et al., 2010), and 1F7 (Gach et al., 2013), (ii) CD4i mAbs A32 (Moore et al., 1994a), and 17b (Thali et al., 1993), (iii) V1–V2-dependent quaternary mAb PG9 (Walker et al., 2009) and (iv) glycan-dependent mAb 2G12 (Rosenberg et al., 2013; Hessell et al., 2009b). Among all mAbs tested, the average reduction in Q461.d1 virus output in the presence of mAb compared to cultures with Den3, a dengue-specific anti-NS1 antibody (Hessell et al., 2009b), ranged from 0–44.5%. MAbs QA255.105 and QA255.157 reduced virus production on average by 43.7% and 40.9%, respectively, which was equivalent to the average ADCVI activity of mAbs with the most impressive activity, 17b (44.5%) and VRC01 (42.3%) (Fig. 3).

To further validate the utility of the results obtained with the RF-ADCC assay, we also tested three mAbs that bound to the VLP, but that did not mediate either neutralizing or RF-ADCC activity. Two of the mAbs tested, QA255.016 and QA255.133 did not mediate ADCVI activity greater than background levels obtained by Den3, and QA255.006 mediated modest ADCVI activity (21.9%) comparable to PG9 (29.9%) and 1F7 (21.1%) (Fig. 3). Overall, the ADCVI assay demonstrated that the mAbs identified with RF-ADCC assay, QA255.105 and QA255.257, demonstrate robust antibody-specific cell cytotoxic

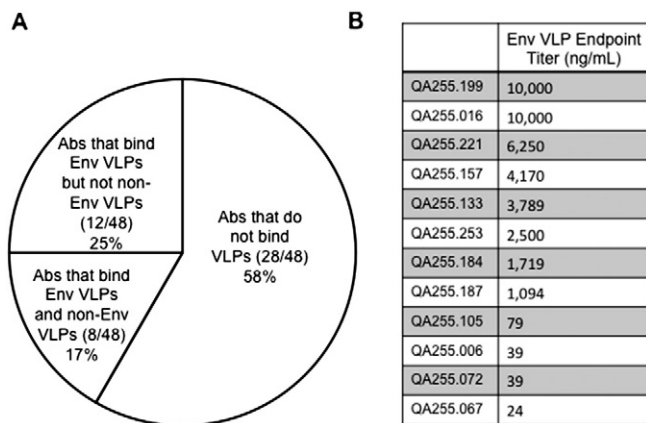


Fig. 1. Specificity and binding properties of mAbs from QA255. (A) The percentage of antibodies that bind only to Env VLPs, both Env and non-Env VLPs or neither VLP are shown in a pie chart. The average endpoint titer for each of the 12 mAbs that bound solely to Env VLPs are recorded in panel (B). Endpoint titers represent the average of two or three independent experiments.

Table 1
V,D and J gene characteristics for QA255 antibodies with neutralizing or ADCC activity.

Antibody	Function	Original isotype	Heavy chain					Light chain						
			V _H gene	Percent mutation from germline ^a	D _H gene	J _H gene	Percent mutation from germline ^b	CDR3 length	Light chain gene	V _L gene	Percent mutation from germline ^a	J _L gene	Percent mutation from germline ^b	CDR3 length
QA255.087	Neutralization	IgG1	1-69	6.94	4-11	6	14.52	28	Kappa	3-20	5.32	2	15.79	9
QA255.105	Both	NA ^c	3-15	9.18	2-21	6	12.9	17	Kappa	3-20	7.45	2	5.26	9
QA255.157	ADCC	IgG1	1-69	7.32	6-13	6	12.9	21	Kappa	3-20	3.94	5	0	8
QA255.187	Neutralization	IgG1	1-69	8.74	2-2	5	5.88	18	Kappa	1-39	9.24	3	8.57	9
QA255.253	ADCC	IgG1	1-69	8.63	3-3	5	3.92	25	Kappa	3D-20	7.8	3	5.26	10

^a Percent mutation at the nucleotide level for the V gene as determined by imgt.org.

^b Percent mutation at the nucleotide level for the J gene as determined by imgt.org.

^c Sequence required to determine isotype was not available (NA) for this antibody.

activity. Additional mAbs that mediate more moderate activity may also be identified with the ADCVI assay.

3.5. Antibody QA255.105 Targeted a V3-specific Linear Epitope

ADCC-mediating antibodies target both linear and discontinuous epitopes on gp41 and gp120, including CD4i epitopes, the CD4bs, and V1–V2 and V3 regions (Pollara et al., 2013; Guan et al., 2013). To determine the epitope specificity for each of the three mAbs, we first tested whether any bound to linear peptides representing the V1 (N-terminal, V1–V2 junction), V2 (N-crown, C-crown, C-terminal) or V3 (N-terminal, C-terminal) regions of Q461.d1 (Blish et al., 2010). None of the antibodies reacted against linear peptides representing the V1 and V2 peptides or the V3 C-terminal region (data not shown). However, mAb QA255.105 demonstrated strong binding with an EPT of 2.4 ng/mL to the N-terminal peptide of V3 as did a V3-peptide specific control, mAb 447-52D (EPT 156 ng/mL, Fig. 4A). To narrow down the

region targeted by mAb QA255.105, we measured antibody binding against 14-mer linear peptides spanning the V3 N-terminal region from the Consensus A peptide sequence (NIH AIDS Repository, Fig. 4B). While QA255.105 did not bind peptides 072, 073 or 075, it demonstrated strong binding with an EPT of 39 ng/mL against peptide 074 (Fig. 4C–F). The V3-specific, positive control mAb 447-52D bound peptide 073 with an EPT of 2500 ng/mL and weakly bound peptide 074 (EPT 10,000 ng/mL) but not peptides 072 or 075 (Fig. 4C–F). These observations are expected based on the accessibility of the 447-52D target epitope within each peptide (Stanfield et al., 2004; Cardozo et al., 2009).

To determine whether QA255.105 V3-specific binding was present as a significant fraction of QA255 plasma, we also examined QA255 plasma IgG from 914 dpi. The plasma IgG bound to the Q461.d1 V3 N-terminus but not the V3 C-terminus peptide (EPT 2500 ng/mL, Fig. 4A and data not shown), supporting the presence of QA255.105-like Abs in this plasma. Additionally, the plasma bound uniquely to Consensus A peptide #074 and did not demonstrate measurable

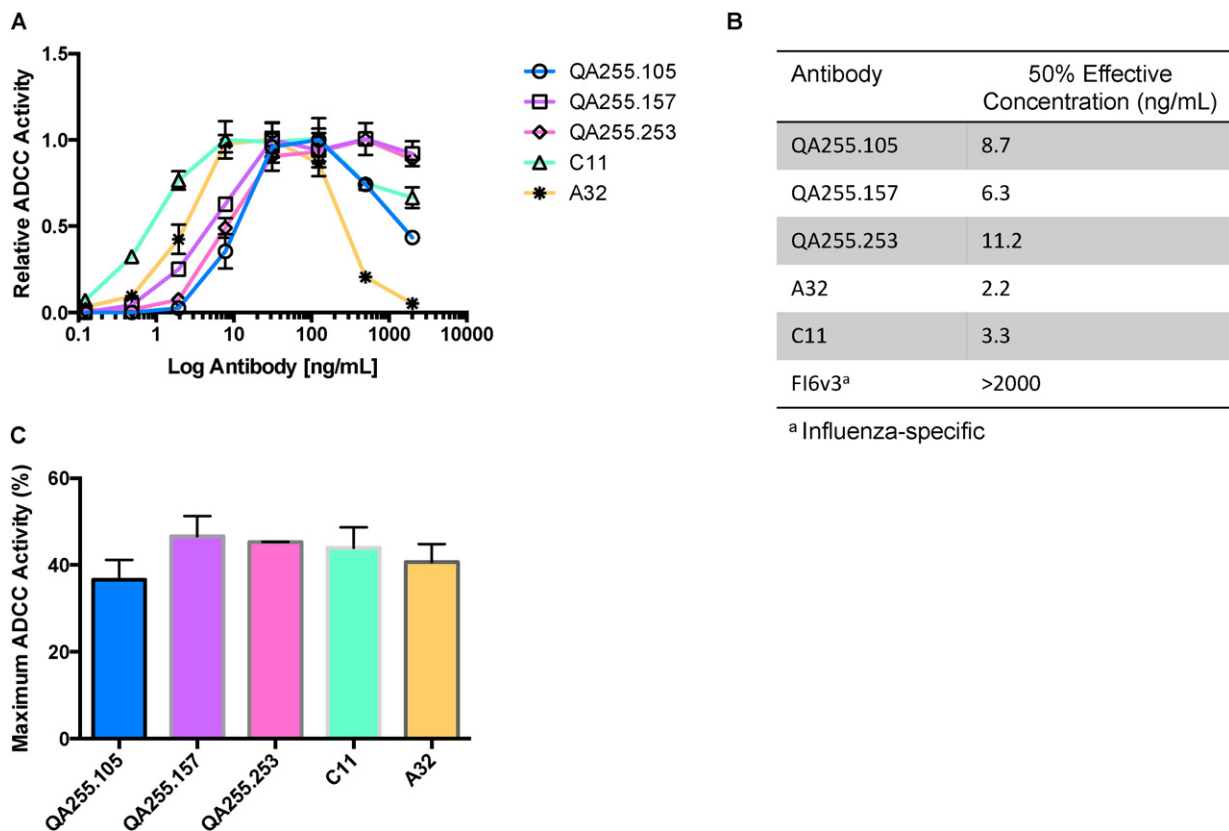


Fig. 2. QA255 mAb ADCC potency. (A) The relative ADCC activity of three QA255 mAbs, A32 and C11 is shown on the y-axis versus log₁₀ antibody concentration (ng/mL) on the x-axis. The average EC₅₀ (ng/mL) value from all experiments is recorded for each mAb as indicated on the x-axis. (B) Maximum percent ADCC activity is reported for each mAb as indicated on the x-axis. Panels A and C are representative data from 3–5 independent experiments and error bars represent the standard deviation from duplicate measurements.

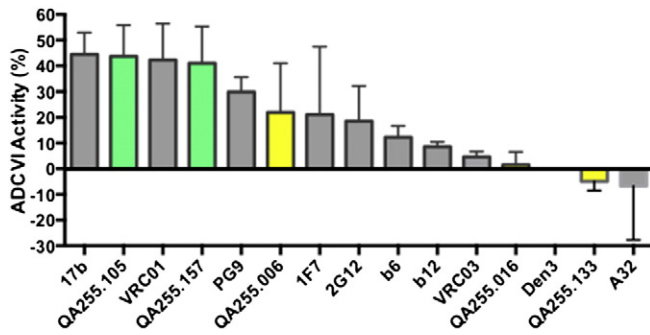


Fig. 3. QA255 mAb ADCVI activity. Percent ADCVI activity for QA255 mAbs and 10 control mAbs is shown on the y-axis. These data are the average of triplicate measurements combined from two independent experiments performed using unique PBMC donors where error bars indicate the standard deviation. MAbs from QA255 that mediate ADCC are shown in green and those that bound VLPs but did not mediate ADCC are shown in yellow. All control mAbs are shown in grey.

binding against the other three peptides (Fig. 4C–F), suggesting that QA255.105-like Abs constitute the majority of QA255 plasma IgG V3-specific binding activity.

3.6. Antibodies QA255.157 and QA255.253 Targeted a CD4-induced, C11-like Epitope

As neither QA255.157 nor QA255.253 bound to any of the linear peptides, we considered the possibility that these Abs targeted a conformationally-dependent epitope. Given their potency, we hypothesized that the Abs may target a CD4-induced epitope. To test this hypothesis, we performed a competition ADCC assay using Fab fragments generated from mAbs A32, C11 and 17b, where A32 and C11 target non-overlapping regions within CD4i Cluster A and 17b targets CD4i Cluster C. While both A32 and 17b Fab inhibited ADCC mediated by the autologous variant with complete effector function by at least 90%, neither Fab inhibited ADCC activity of any of the three antibodies (Fig. 5A, B). In contrast, C11 Fab, when tested at a concentration resulting in 69% average inhibition of autologous ADCC activity, was capable of inhibiting between 83% and 88% of QA255.157 and QA255.253 ADCC activity, respectively (Fig. 5C). The ADCC activity of the V3-specific Ab QA255.105 was unaffected by pre-incubation with A32, 17b or C11 Fab (Fig. 5A–C). C11-like antibody activity was confirmed when pre-incubation of Q461.d1 gp120-coated plates with serial dilutions of Abs QA255.157 or QA255.253 inhibited binding of biotinylated, full-length C11 antibody (Bt-C11) by 80% and 72%, respectively, at the highest concentration tested, suggesting that the binding and inhibition pattern between QA255.157, QA255.253 and C11 is reciprocal. As observed in the Fab blocking experiments, pre-incubation with Ab QA255.105 did not inhibit Bt-C11 binding (Fig. 5D).

3.7. CD4-induced, C11-like Antibodies Demonstrated Strong Cross-clade ADCC Activity

In addition to potency, breadth is likely a critical characteristic of a protective ADCC-mediating antibody due to the extensive diversity of HIV. Because the cross-clade ADCC breadth of CD4-induced antibodies remains incompletely defined, we evaluated ADCC activity of the two C11-like Abs and the one V3-specific Ab identified here against a panel of 11 gp120 proteins from clades A, B, C, A/D and C/D. We also examined the prototype CD4i Ab C11 as the breadth of this mAb has not been well characterized. The envelope panel encompassed 3 clade A gp120s, including the QA255 autologous gp120 obtained 21 days post infection, 3 clade B, 2 clade C, 1 clade A/D and 1 clade C/D gp120 and SIVmac239 gp120 as a negative control. Because PBMC donor variability can affect percent ADCC killing, we have reported the average

of experimental duplicates from two separate donors. Two of the three antibodies tested, QA255.157 and QA255.253, as well as CD4i mAb C11, demonstrated ADCC activity against all clade A and A/D gp120s, both clade C and C/D gp120s and two of three clade B gp120s using PBMCs from both donors (Fig. 6). Neither mAbs QA255.157, QA255.253 nor C11 could mediate activity against cells coated with clade B YU2 gp120. Antibody QA255.105 demonstrated consistent ADCC activity against clade A gp120 BG505, clade A/D gp120 BL035 and one clade C gp120 CAP210. None of the antibodies demonstrated measurable activity against SIVmac239 gp120-coated CEM cells (Fig. 6).

3.8. QA255.17 and QA255.253-like Abs Contributed to QA255 Plasma-mediated ADCC Activity

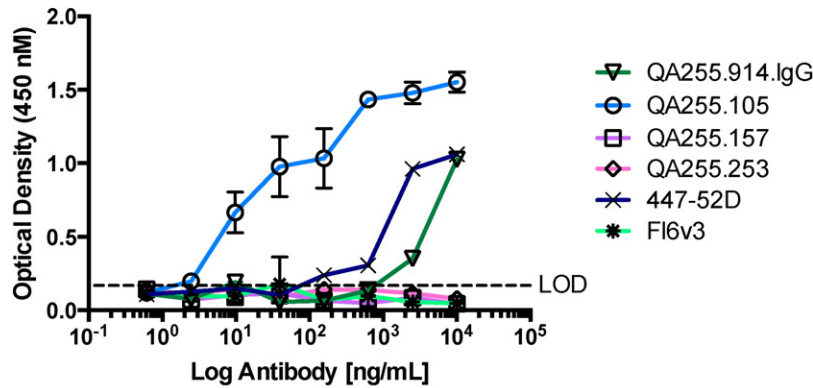
To determine whether the mAbs we isolated accounted for the breadth of ADCC activity in QA255, we screened QA255 plasma IgG obtained 914 dpi – the same time point at which the mAbs were isolated – against the panel of 11 envelopes. The dilution used for QA255 plasma IgG was identified as the concentration resulting in peak ADCC activity against cells coated with Q461.d1 and QA255.21P.A17 gp120s (Fig. S1). Similar to Abs QA255.157 and QA255.253, QA255 plasma IgG mediated consistent ADCC activity against two of three clade A gp120s, all three clade B gp120s, both clade C gp120s as well as Clades A/D and C/D gp120 proteins (Fig. 7A). Modest ADCC activity was detected against the third clade A gp120 protein, BG505, using one of two PBMC donors (Donor 2). Overall, mAbs QA255.157 and QA255.253 mediated activity against 10 of the 11 gp120s targeted by QA255.914 dpi plasma.

Because QA255.157 and QA255.253 recapitulated the majority of QA255 plasma breadth, we next addressed whether QA255.105 or QA255.157-like antibodies contributed to QA255 plasma potency and how quickly these mAbs may have developed following infection. To address this question, we generated a LALA mutation in the CH2 region of all three antibodies to eliminate binding to FcRs and complement (Hezareh et al., 2001; Shields et al., 2001) and tested whether these LALA variants could inhibit QA255 plasma IgG isolated from 6 time points ranging from 89 dpi to 914 dpi. As expected, introduction of the LALA change did not affect binding to Q461.d1 gp120 protein (Fig. S2A) but did abrogate ADCC activity at all Ab concentrations tested (Fig. S2B). Additionally, pre-incubation of Q461.d1 Env-coated target cells with each of the LALA variants resulted in almost complete inhibition of autologous Ab-mediated ADCC activity but had no effect on Ab A32 ADCC activity thus demonstrating the LALA Abs' specificity (Fig. S2C & S2D). When the LALA variants were used in competition with QA255 plasma IgG, pre-incubation with QA255.157 LALA reduced ADCC activity by 23–47% across all time points beginning at 189 dpi. Similarly, pre-incubation with QA255.253 LALA inhibited ADCC activity by 15–38%. Neither LALA variant reduced ADCC activity at the first 89 dpi time point despite measurable ADCC activity (Fig. 7B). Pre-incubation with QA255.105 LALA had no effect on QA255 914 dpi plasma IgG ADCC activity (Fig. S2D, right panel). Thus, these data indicate that C11-like Abs developed within the first six months following infection and were maintained through at least 914 dpi.

3.9. QA255.157 and QA255.253-like ADCC Activity is Common in HIV Infection

Little is known about whether ADCC-mediating, C11-like antibodies routinely contribute to ADCC activity. To determine how commonly these responses develop following HIV infection, we examined plasma obtained ~3 years post infection from 10 chronically infected individuals from the Mombasa cohort for ADCC-mediating antibodies targeting the same C11-like epitope. Plasma from 9 of the 10 women demonstrated measurable ADCC activity. Among the 9 women, QA255.157- and QA255.253-like antibodies contributed between 29–75% and 18–78%, respectively, of total ADCC

A Q461.d1 V3 N terminus



B

Q461.d1 V3

Consensus A Peptide #072

Consensus A Peptide #073

Consensus A Peptide #074

Consensus A Peptide #075

CIRPGNNTRKSVRIGPGQAFYATGDDITGDIRNAHC

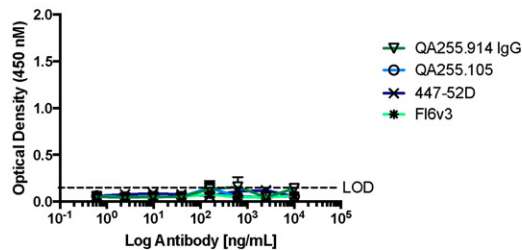
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N-----G-----

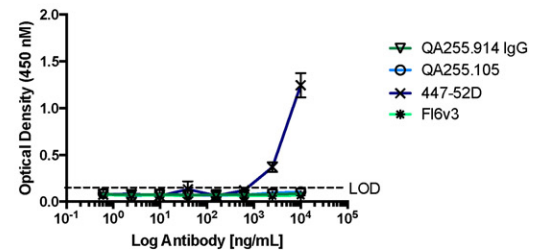
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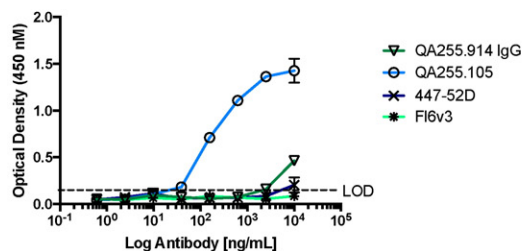
C Consensus A Peptide #072



D Consensus A Peptide #073



E Consensus A Peptide #074



F Consensus A Peptide #075

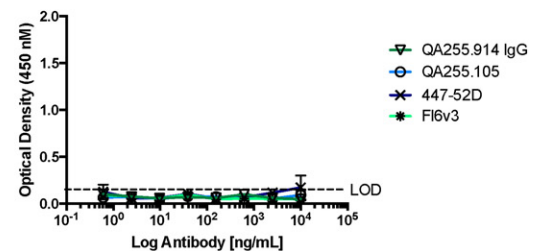


Fig. 4. QA255 mAb binding to V1, V2 and V3 linear peptides. Panel (A) shows Q461.d1 V3-N terminal peptide binding expressed as optical density measured at 450 nm on the y-axis versus \log_{10} antibody concentration (ng/mL) on the x-axis. These data are representative of two independent experiments where each antibody was run in duplicate and error bars indicate the standard deviation. (B) Alignment of Q461.d1 and Consensus A V3 peptides. Q461.d1 V3 N-terminal linear peptide (22-mer) is colored in pink, V3 C-terminal linear peptide (22-mer) is colored in blue and the overlap contained in both peptides colored in purple. Residue changes between the Consensus A Peptide sequence and Q461.d1 V3 peptide are noted for each of the four linear peptides. (C–F) Binding to 14-mer Consensus A peptides #072 (C), #073 (D), #074 (E), #075 (F), expressed as optical density measured at 450 nm, is shown on the y-axis relative to \log_{10} antibody concentration (ng/mL) on the x-axis. In all panels, data are representative of two independent experiments and error bars indicate the standard deviation from duplicate measurements. The limit of detection (LOD) is shown as a dotted line.

activity (Fig. 7C). Taken together, these data suggest that among ADCC-mediating antibodies, C11-like antibodies are routinely produced and may account for as much as 78% of an individual's ADCC-mediating antibody repertoire.

3.10. QA255.157 and QA255.253 Originated From Unique B Cells

Gene usage for the five antibodies identified in this study demonstrated patterns compatible with previous reports (Bonsignori et al., 2012;

Hicar et al., 2010; Scheid et al., 2011; Stanfield et al., 2004; Li et al., 2012). Four of the five neutralizing and ADCC-mediating antibodies were encoded by IgV_H gene V_H1-69. Ab QA255.105, which mediated both modest neutralizing and ADCC activity, was instead encoded by IgV_H gene V_H3-15, as are other V3-specific antibodies, including Ab 447-52D (Stanfield et al., 2004). Despite being encoded by the same V_H gene, both ADCC-mediating Abs QA255.157 and QA255.253 that targeted the same epitope use different D_H and J_H genes and have unique CDR3 regions, suggesting that they originated from different B

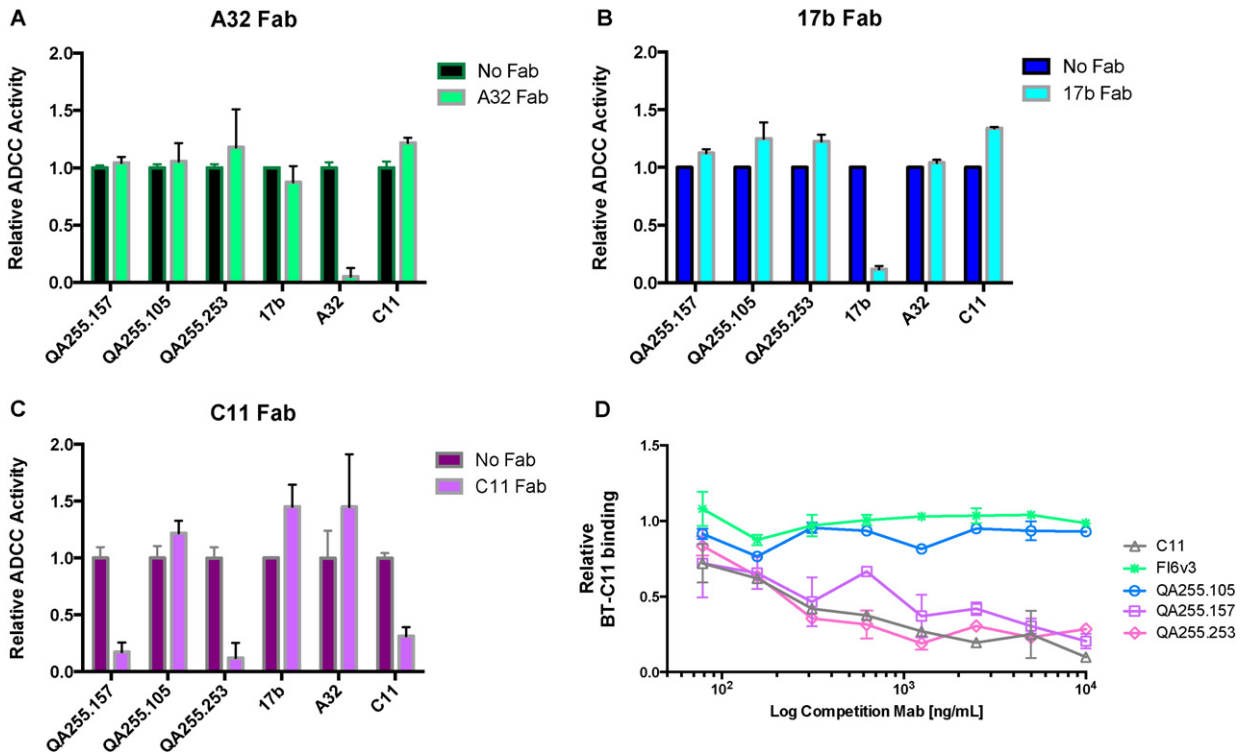


Fig. 5. CD4i Fab inhibition of QA255 mAb ADCC activity. (A–C) Relative ADCC activity for mAbs specified on the x-axis is shown in the presence (lightly shaded bars) and absence (darker shaded bars) of A32 Fab (A, green), 17b Fab (B, blue) and C11 Fab (C, purple). Data represent the average of two independent experiments and error bars indicate the standard deviation from duplicate measurements. (D) Relative Bt-C11 activity is expressed on the y-axis versus \log_{10} antibody concentration (ng/mL) of the competitor mAb on the x-axis. Data are representative of two independent experiments and error bars indicate the standard deviation from duplicate measurements.

cells (Table 1). Thus, these data provide evidence that the epitope stimulating development of these C11-like antibodies is able to induce similar antibody production from B cells of unique clonal lineages. The percent mutation from the predicted germline sequence was 9.2%, 7.3% and 8.6% for QA255.105, QA255.157 and QA255.253 respectively (Table 1), consistent with previous observations for ADCC-mediating and weakly neutralizing Abs but in contrast to the significant somatic hypermutation observed with many bnAbs such as the VRC01/VRC03, CH30–CH34 and PGT families (reviewed in (Corti and Lanzavecchia, 2013)).

4. Discussion

A detailed understanding of the epitope specificity of ADCC-mediating antibodies is a pre-requisite for developing effective immunization strategies that optimize protection by these antibodies. In comparison to the wealth of data characterizing bnAbs, surprisingly little is known about the characteristics defining a broad and potent ADCC-mediating immune response. In this study, we describe a method to identify memory B cells producing ADCC-mediating antibodies from HIV-infected individuals. We applied this method to isolate mAbs from an individual with a cross-clade ADCC and neutralizing Ab response (Bosch et al., 2010). The three ADCC antibodies identified here mediate potent ADCC activity, and competition studies suggest that two target a CD4-induced epitope that resembles the epitope bound by the C11 antibody. Subsequent analysis determined that these two mAbs, QA255.157 and QA255.253, demonstrated impressive cross-clade breadth, mediating activity against 10 of 11 envelopes tested from clades A, B and C. When expressed as LALA variants, Abs QA255.157

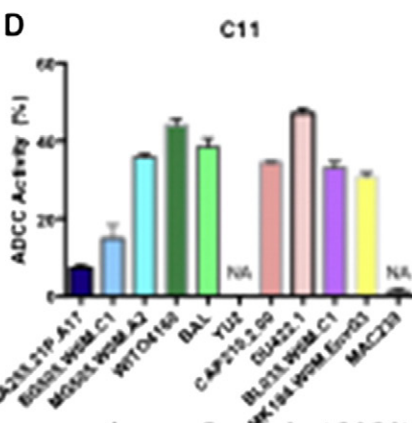
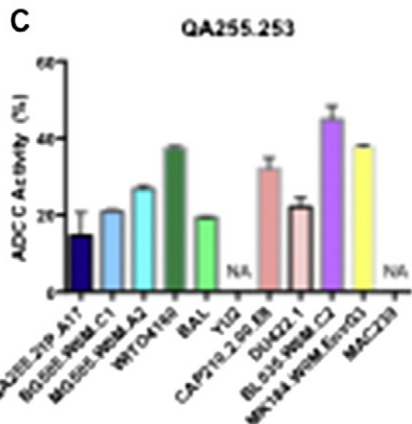
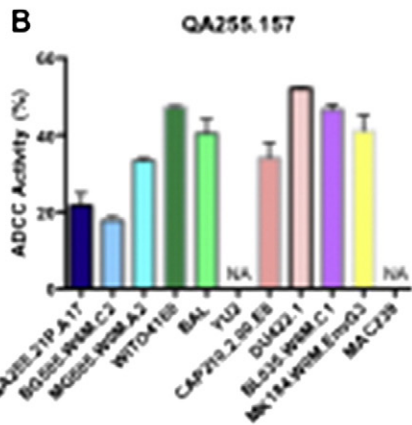
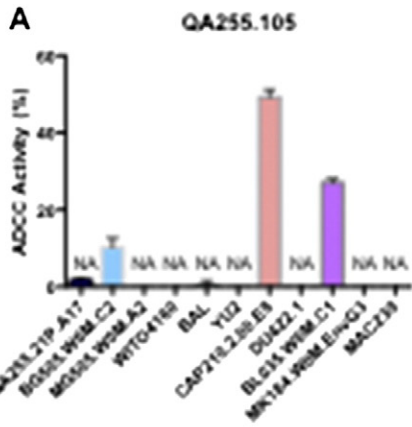
and QA255.253 inhibited QA255 plasma IgG activity consistently beyond 189 dpi. Thus, these data provide evidence that CD4i antibodies that target an epitope also bound by mAb C11 constitute a substantial ADCC response and that these antibodies contribute to ADCC activity within the first six months of infection.

Previous reports using VLP screening strategies identified 0.2 to 1.8% of PBMCs as HIV-specific B cells (Hicar et al., 2010; Li et al., 2012). Similarly, we identified 1.44% of CD19+, CD27+ cells to bind VLPs. Among the 192 VLP-binding B cells, functional antibodies were rescued from 48, 12 of which were confirmed to be HIV-envelope specific. Surprisingly, 8 of 48 antibodies were specific for Gag p24 and bound to both the non-Env VLP and Env VLPs. The isolation of Gag-specific mAbs may be the result of some low level Gag protein on the VLP surface or some partial disruption of the lipid envelope of the purified VLPs. Further characterization of the functional activity of anti-gag specific antibodies may be warranted, as early reports have suggested that Gag proteins may accumulate on the surface of infected cells (Ikuta et al., 1989; Nishino et al., 1992) though anti-p24 ADCC activity has been sparsely studied to date (Grunow et al., 1995).

The assay used to define the breadth and potency of these ADCC-mediating antibodies is particularly relevant because it has been shown to measure activity that correlates with protection in multiple studies, including both human cohorts (Milligan et al., 2015; Mabuka et al., 2012) and macaques (Gómez-Román et al., 2005; Hidajat et al., 2009; Xiao et al., 2010; Barouch et al., 2012). Although the relevance of this assay for predicting important outcomes is relatively well established compared to other ADCC assays, the mechanistic basis for killing in the RF-ADCC assay remains somewhat poorly defined. All

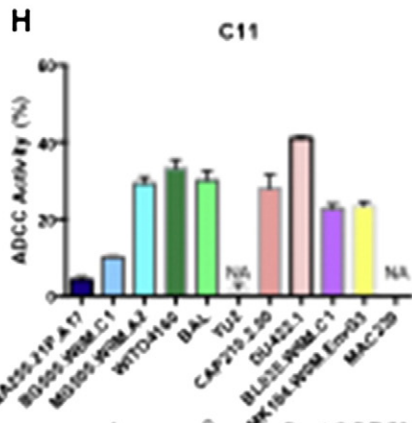
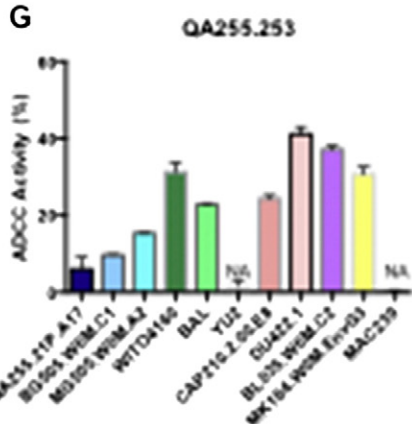
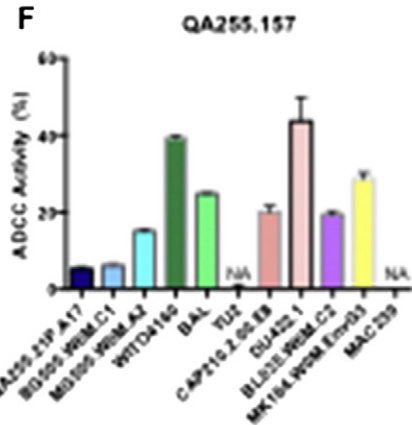
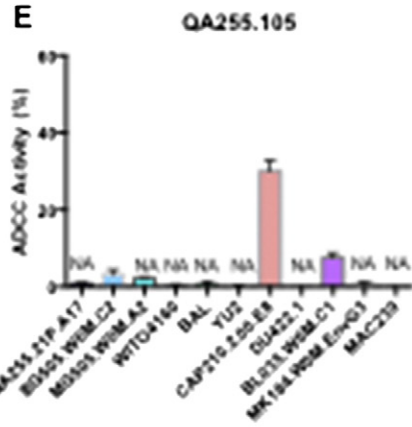
Fig. 6. QA255 mAb ADCC breadth. ADCC activity of mAbs indicated at the top of each panel was assessed using gp120s from three Clade A (blue), three Clade B (green), two Clade C (pink), one Clade A/D (purple), one Clade C/D (yellow) and one SIV-macaque gp120 variant(s) as specified on each x-axis. Percent ADCC activity is shown on the y-axis. Data are reported using PBMCs from two unique donors (Donor 1 & Donor 2) and are an average of duplicate measurements with error bars indicating the standard deviation. 'NA' indicates that the antibody did not mediate ADCC activity against the specific gp120 protein.

Donor 1



Clade A B C ADCC50/

Donor 2



Clade A B C ADCC50/

three antibodies, irrespective of epitope specificity, demonstrated comparable potency in the RF-ADCC assay to Abs A32 and C11 – two Abs identified as the most potent ADCC Abs in a study of 41 CD4i-specific Abs (Guan et al., 2013). Both the CD4i-specific and V3-specific mAbs isolated here mediated ADCVI activity that was superior to A32 and most other mAbs known to mediate ADCVI, indicating potent inhibitory activity against replicating virus. Interestingly, the two mAbs that targeted the C11-like epitope, QA255.157 and QA255.253, demonstrated weak binding to the enveloped VLP. Prior studies have shown that CD4i-directed mAbs capable of ADCC generally bind poorly to the soluble envelope trimer (Moore et al., 2006; Tong et al., 2013; Ray et al., 2014) presumably due to epitope inaccessibility in the absence of cellular CD4 (Acharya et al., 2014; Veillette et al., 2014; Pancera et al., 2010). Nonetheless, these mAbs showed activity in the ADCVI assay where reduction in virus outgrowth is the result of blocking virus spread, thus suggesting these mAbs can indeed target trimer-expressing cells. Given the heterogeneity observed within envelope variants present on the surface of virions and VLPs alike (Moore et al., 2006; Tong et al., 2013; Poirnard et al., 2003), it remains to be determined what envelope structure bound to the BCRs encoding the CD4i Abs identified in this study.

Because median genetic variation within the Env glycoprotein is estimated to be 25% between subtypes (Korber et al., 2001; Gaschen et al., 2002), ADCC-mediating antibodies must be able to target cells infected with genetically distinct viruses in order to adequately prevent infection. Thus, antibody breadth is likely to be important for a protective ADCC-mediating response. Recent data has suggested that HIV controllers demonstrated ADCC activity against a broader range of envelopes (median of 8 different gp120 proteins) than HIV progressors (median of 1 gp120) (Madhavi et al., 2014). Of the 10 envelope proteins we tested, Abs QA255.157, QA255.253 and C11 were able to mediate activity against all of the gp120-coated target cells except the cells coated with one clade B virus, YU2. These latter data were unexpected as C11 binding to both YU2 gp120 monomer (Kwong et al., 2002) and CD4-bound YU2 trimer expressed on cells have been reported (Veillette et al., 2014). It is unclear whether these discrepancies reflect differences in assay conditions or in the protein preparations tested. When compared with data from QA255.914 dpi plasma IgG, QA255.157 and QA255.253 were each able to recapitulate 91% of ADCC breadth. Interestingly, a number of gp120 proteins against which both CD4i antibodies were able to mediate activity included Tier 2 viruses BG505.W6M.C2 (clade A), WITO4160 (clade B), DU422.1 (clade C) and Cap210.2.00.E8 (clade C) that are less susceptible to neutralizing antibodies. Few reports have tested large-scale panels of envelopes to determine the ADCC breadth of either mAbs or infected plasma. Of the antibodies produced from vaccine recipients, only one of 21 mAbs were able to mediate ADCC activity against four chronically infected cell lines expressing envelope from distinct HIV clades (Bonsignori et al., 2012). Thus, the study presented here provides strong evidence that mAbs targeting CD4i epitopes can mediate broad cross-clade ADCC activity, suggesting that such antibodies could be valuable as part of a vaccine response.

The third ADCC-mediating antibody identified through our sorting strategy, QA255.105, targets the N-terminus of V3. This Ab exhibits stronger binding to Env VLPs, comparable ADCC potency but less breadth in comparison to the two CD4i Abs. Our data coincides with data reported in Bonsignori et al. (2012), where they also identified one V3-specific Ab from a vaccine recipient (Bonsignori et al., 2012). This Ab similarly demonstrated strong ADCC potency with limited breadth and weak nAb activity. Because the V3 N-terminal region is a fairly heterogeneous region of epitope specificity, it is perhaps not surprising that Ab QA255.105 displayed limited ADCC breadth.

A32-like ADCC responses have been reported to comprise at least 50% of the ADCC-mediating antibody response in most chronically infected and vaccinated individuals (Ferrari et al., 2011; Bonsignori et al., 2012). Here we observed that most individuals, including

QA255, also produce Abs similar to C11 that target a distinct CD4i epitope, suggesting that a combination of Abs with different CD4i specificities may be contributing to plasma-mediated ADCC activity. Both A32 and C11 recognize discontinuous epitopes within close proximity to each other – specifically A32 to the conformational interface between gp120 and gp41 (Guan et al., 2013; Acharya et al., 2014; Pancera et al., 2010; Finzi et al., 2010) and C11 to the seven-stranded B-sandwich and N- and C-terminus of gp120 (Guan et al., 2013; Pancera et al., 2010; Gohain et al., 2015). Because of their close proximity, both epitopes are found on the electronegative face of gp120 that is obscured by gp41 and only becomes exposed subsequent to CD4 binding via HIV entry or cell-to-cell fusion (Guan et al., 2013; Finnegan et al., 2001; Mengistu et al., 2015). Recent observations using fluorescence correlation spectroscopy have identified that both A32- and C11-like epitopes become exposed within minutes of incubation of HIV and target cells, further validating that these CD4i epitopes are opportune targets of the humoral immune response at extremely early stages of HIV infection (Mengistu et al., 2015). Further studies to identify differences in epitope conformation that result in the production of A32-like or QA255.157- and QA255.253-like antibodies therefore may be useful in guiding effective vaccine design.

Vaccination strategies designed to induce consistent production of bnAbs currently face a number of concerns, including the length of time required to develop the Abs, the amount of somatic hypermutations required to develop strong neutralizing activity, and the relative rarity of B cells capable of inducing bnAb progenitors. We, and others, have clearly demonstrated that ADCC activity can be measured within the first few months following HIV infection in humans, and as early as 3 weeks in some NHP studies (Alpert et al., 2012; Dugast et al., 2014a; Chung et al., 2011b). Regarding QA255.157 and QA255.253, the amount of somatic hypermutation recorded for the two CD4i Abs was <10%, similar to that reported from ADCC-mediating Abs identified from RV144 recipients (Bonsignori et al., 2012), but significantly less than the 21% to 37% required for potent bnAbs such as the CD4bs-specific VRC and CH30-34 (Wu et al., 2011; Wu et al., 2010) and the complex V3 glycan-specific PGT families (Walker et al., 2011; Mouquet et al., 2012). Finally, both QA255.157 and QA255.253 were derived from V_H1-69 genes but use distinct D_H, J_H and light chain genes, implying that each antibody originated from a unique B cell, rather than somatic variants of the same B cell. Taken together, these observations suggest that development of a strong ADCC-specific Ab response may be less affected by the challenges associated with development of a robust bnAb response.

In conclusion, we propose that VLPs that express multiple forms of envelope protein may be a novel method for identifying memory B cells producing ADCC-mediating antibodies. We have found that two Abs with broad and potent ADCC activity originated from unique B cells and showed less mutation from germline than bnAbs. These observations, along with our finding that these responses can be detected within six months of infection and are common among HIV-infected individuals, suggest that this epitope is a versatile target for HIV-specific ADCC-mediating antibodies. Further, eliciting such broadly reactive ADCC Abs may not pose some of the challenges associated with the development of bnAbs. Because ADCC-mediating antibodies target infected cells, therapeutic vaccine strategies designed specifically to elicit ADCC-mediating Abs may be helpful in augmenting approaches to reduce viral reservoirs. Future studies streamlining methods to characterize both the host and viral factors governing the development of ADCC-mediating antibodies will be critical in guiding vaccine strategy to elicit broad ADCC-mediating antibodies.

Funding

The study was funded by R01 AI103981 and R37 AI038518 to JO, AI111863 to PS, R01 AI102715 to DNF, T32 AI07140 to KLW, T32 CA080416 to AD and T32 AI083203 to VC. No author received

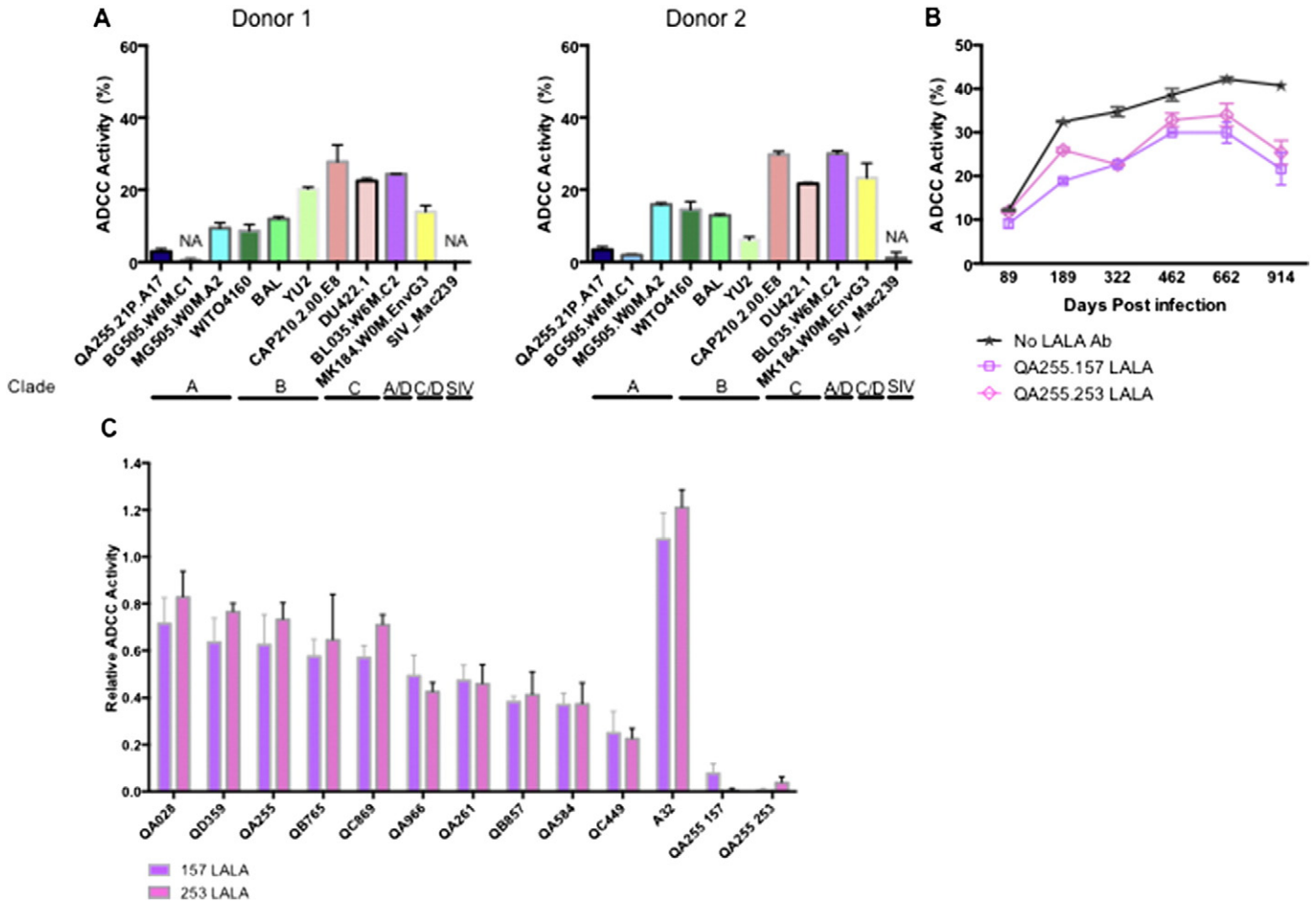


Fig. 7. QA255 914 dpi plasma ADCC breadth and epitope specificity. (A) QA255 plasma IgG (914 dpi) ADCC activity is reported versus the Clade A (blue), B (green), C (pink), A/D (purple), C/D (yellow) and SIV gp120 variant(s) indicated on the x axis and shown in Fig. 6. Percent ADCC activity is reported using PBMCs from two unique donors (Donor 1 & Donor 2) and represents the average of duplicate measurements with error bars indicating the standard deviation. (B) Percent ADCC activity of QA255 plasma IgG isolated from 6 time points as specified on the x-axis is shown in the presence of QA255.157 LALA Ab (purple), QA255.253 LALA Ab (pink) or absence of competitor LALA Ab (gray). Data are representative of two independent experiments and error bars indicate the standard deviation from duplicate measurements. (C) Relative ADCC activity for 10 unique plasma samples when incubated with either QA255.157 LALA (purple) or QA255.253 LALA (pink) is shown on the y-axis. Data are the average of duplicate measurements combined from two independent experiments where error bars indicate the standard deviation.

compensation by a pharmaceutical company or other agency for completion of this manuscript.

Conflict of Interest

There are no conflicts of interest associated with this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Study design: KW, VC, DNF and JO. Performed the experiments: KW, VC, ASD, SR, JFW, JSG. Analyzed data: KW, VC, AD, JSG. Contributed reagents: XC, PS. Wrote the paper: KW, JO with input from all authors.

Acknowledgments

The authors thank the clinic, laboratory, and administrative staff of the Mombasa cohort study for all their efforts. We also gratefully acknowledge the women who participate in the Mombasa cohort, and we are particularly indebted to the individual who provided the samples for this study. We also thank Hieu Nguyen for help performing the VLP sorting experiments and Keshet Ronen for reviewing the manuscript.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2015.09.001>.

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