

# Coordinated Fc-effector and neutralization functions in HIV-infected children define a window of opportunity for HIV vaccination

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**Objectives:** Antibody function has been extensively studied in HIV-infected adults but is relatively understudied in children. Emerging data suggests enhanced development of broadly neutralizing antibodies (bNAbs) in children but Fc effector functions in this group are less well defined. Here, we profiled overall antibody function in HIV-infected children.

**Design:** Plasma samples from a cross-sectional study of 50 antiretroviral therapy-naive children (aged 1–11 years) vertically infected with HIV-1 clade A were screened for HIV-specific binding antibody levels and neutralizing and Fc-mediated functions.

**Methods:** Neutralization breadth was determined against a globally representative panel of 12 viruses. HIV-specific antibody levels were determined using a multiplex assay. Fc-mediated antibody functions measured were antibody-dependent: cellular phagocytosis (ADCP); neutrophil phagocytosis (ADNP); complement deposition (ADCD) and natural killer function (ADNK).

**Results:** All children had HIV gp120-specific antibodies, largely of the IgG<sub>1</sub> subtype. Fifty-four percent of the children exhibited more than 50% neutralization breadth, with older children showing significantly broader neutralization activity. Apart from ADCC, observed only in 16% children, other Fc-mediated functions were common (>58% children). Neutralization breadth correlated with Fc-mediated functions suggesting shared determinants of enhanced antibody function exist.

**Conclusions:** These results are consistent with previous observations that children may develop high levels of neutralization breadth. Furthermore, the striking association between neutralization breadth and Fc effector function suggests that HIV vaccination in children could yield multifunctional antibodies. Paediatric populations may therefore provide an ideal window of opportunity for HIV vaccination strategies.

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## Introduction

Broadly neutralizing antibodies (bNAbs) to HIV-1 are of particular interest for vaccine-mediated humoral immunity. Studies in macaque models [1–4], humanized mice [5] and humans [6] have provided proof-of-principle that a vaccine capable of inducing these types of antibodies is likely to be effective against most circulating HIV strains. However, only a small proportion of HIV-infected individuals generate bNAbs [7] and no HIV vaccine candidate has been able to elicit antibodies with sufficient breadth [8,9].

Antibody functions have largely been described in adults but less is known for children despite their distinct course of HIV infection. AIDS typically develops more rapidly in paediatric HIV cases compared with adults [10–12] and infants and children rapidly develop broader and more potent neutralizing antibodies than adults [13–15]. Furthermore, an isolated bNAb from an infant showed low somatic hypermutation and lacked insertions and deletions typical of bNAbs from adults, suggesting that infants may have a more direct pathway to breadth that does not require years of affinity maturation [13].

Antibodies are, however, multifunctional and their antiviral activity results from the synergistic functions of the fragment antigen binding (Fab) and fragment crystallizable (Fc) regions [16,17]. Antibodies capable of mediating Fc-effector functions via innate immune cells are commonly identified in HIV-infected people, and may contribute to viral control [18,19] and to slowing HIV acquisition [20]. Additionally, elite controllers may have more Fc-mediated antibody polyfunctionality that recruits a more coordinated innate immune response [21]. In the only HIV vaccine trial that has shown any protection to date, lower risk of HIV acquisition was associated with Fc-mediated antibody functions [22]. Fc-mediated functions also contribute to optimal antiviral activity for some bNAbs [23]. bNAbs were shown to interfere with the establishment of a silent reservoir through Fc-Fc receptor-mediated mechanisms [24]. Furthermore, reduced protection by passively administered bNAbs occurred when Fc-receptor activity was engineered out of these antibodies [25]. In infected adults, HIV-specific Fc-effector functions early in HIV infection predicted the downstream development of bNAbs [26] but such studies have not been performed in children.

Understanding the development of broadly neutralizing and Fc-mediated antibody functions in paediatric natural HIV infection remains important. To address this, we assessed the presence, magnitude and correlation of neutralizing and Fc-mediated functions in a cross-sectional study of 50 antiretroviral therapy (ART)-naive, chronically infected children aged 1–11 years, predominantly infected with HIV clade A. Fifty-four percent of the children developed neutralization breadth against the

panel of viruses used in this study. There was a significant association of antibody neutralization breadth with the Fc-mediated functions suggesting common determinants of function. These results agree with the accumulating findings that children may have unique immunological profiles that favour the development of more effective antibodies and understanding of these profiles may inform vaccine strategy.

## Materials and methods

### Ethics statement

The study used samples from a previously reported parent study [27]. Ethical approval was received from the Kenya Medical Research Institute Science and Ethics Review Unit (SERU-3530). Informed consent for study participation and sample storage was obtained from each child's parent/guardian.

### Study population

Recruitment of study participants is reported elsewhere [27]. In summary, HIV-infected children aged 1–11 years were recruited into a cross-sectional study between October 2010 and May 2012 during care or first visit to the Kilifi County Hospital, in Coastal Kenya, where HIV-1 clade A is predominant. At the time of recruitment, the WHO's treatment guidelines recommended: all those less than 24 months: combination ART (cART); 25–59 months: cART if CD4<sup>+</sup> T-cell percent less than 25% and/or if in WHO clinical stage 3 or 4; greater than 60 months: cART if their CD4<sup>+</sup> T-cell percent less than 20% and/or if in WHO clinical stage 3 or 4 [28]. Of the 121 children invited for study participation, guardians/parents of 50 children self-reported that the child had not initiated ART, and these children were included in this study.

### Determination of HIV-specific antibody neutralization breadth

Antibody neutralization was determined against a globally representative panel of 12 viruses belonging to subtypes A ( $n = 1$ ), B ( $n = 2$ ), C ( $n = 3$ ), G ( $n = 1$ ) and circulating recombinant forms (CRFs) ( $n = 5$ ) [15,29]. Neutralization was measured as previously described [30–33] by a reduction in luciferase gene expression after single-round infection of TZM-bl cells with Env-pseudotyped viruses. Antibody potency was calculated as the plasma dilution needed to neutralize 50% viral infectivity, and breadth as the ability to neutralize more than 50% of this multisubtype panel.

### Determination of HIV-specific antibody subclass levels

#### *Multiplex assay*

A previously described multiplex assay was adapted [34], whereby microsphere beads were coupled to HIV

antigens clade A Q461.D1 gp120, clade A BG505 SOSIP, clade A 92RW020 gp140, clade A 94UG103 gp120, clade B JRFL gp140, clade B MN gp120, clade C TV-1 gp140 and clade C IAVIC22 gp120, and two control antigens HA (A/California/07/2009), HA (A/Victoria/3/75) and inactivated Tetanus toxoid. Coupled beads were incubated with diluted plasma overnight at 4 °C and the levels of total IgG and IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgA<sub>1</sub> and IgA<sub>2</sub> detected by PE-conjugated detection agents (Southern Biotech, Birmingham, Alabama, USA) using an IQue Screener Plus (IntelliCyt).

#### ELISA

In brief, 50 µg of the recombinant BG505 clade A gp120 (donated by Elise Landais) were used to coat plates prior to incubation with diluted test plasma. Subclass-specific antihuman immunoglobulin conjugated to alkaline phosphatase (mouse anti-IgG, anti-IgG<sub>1</sub>, anti-IgG<sub>2</sub>, anti-IgG<sub>3</sub>, anti-IgG<sub>4</sub> or goat anti-IgA) from Southern Biotech, USA were used for antibody subclass detection. Relative arbitrary antibody units were calculated by interpolating OD readings to standard curves prepared using pooled hyper-reactive HIV-positive plasma.

### Determination of HIV-specific Fc-mediated antibody functions

#### *Antibody-dependent phagocytosis*

Fluorescent neutravidin yellow-green beads (Invitrogen, Waltham, Massachusetts, USA) were coated with AVI-tag biotinylated clade A gp120 (donated by Devin Sok and Joseph Jardine). The ability of patient plasma samples to generate antigen-specific immune-complexes that a) drive their uptake by a monocytic cell line (THP-1; ATCC TIB-202), to establish antibody-dependent cellular phagocytosis (ADCP), b) drive their uptake by neutrophils enriched from healthy donor's whole blood, to establish antibody-dependent neutrophil phagocytosis (ADNP), measured by flow cytometry [21,35,36]. Neutrophils were defined as CD3<sup>-</sup>CD14<sup>-</sup>CD66b<sup>+</sup> cells (antihuman CD3 AF700 and CD14 APC-Cy7 (BD Biosciences, San Jose, California, USA) and antihuman CD66b Pacific Blue (BioLegend, San Diego, California, USA)], whereas THP-1 cells were left untreated. The phagocytic activity was presented as a score calculated as the [(% cells that have taken up antigen-coupled beads) × (MFI (mean fluorescent intensity) of cells that have taken up antigen-coupled beads)]/10000.

#### *Antibody-dependent complement deposition*

Fluorescent neutravidin red beads (Invitrogen) were coupled with AVI-tag biotinylated clade A gp120 and then incubated with diluted patient plasma, prior to adding low-tox guinea pig complement (Cedarlane, Burlington, North Carolina, USA). Antibody activation of the complement resulted in release of C3, which was measured using antihuman C3 FITC goat IgG (BD Biosciences) by flow cytometry.

#### *Antibody-dependent natural killer cell degranulation*

In brief, plates were coated with AVI-tag gp120, blocked, and patient plasma added before incubating for 2 h at 37 °C. NK cells isolated from a healthy donor's whole blood were added simultaneously with anti-CD107a PE-Cy5 (BD Biosciences), Brefeldin A (Sigma) and Golgi Stop (BD Biosciences) and incubated for 5 h at 37 °C as previously reported [37]. NK cells were then stained with anti-CD56 PE-Cy7, anti-CD16 allophycocyanin (APC)-Cy7 and anti-CD3 Alexa Fluoro 700 (BD Biosciences), fixed (FIX&PERM cell fixation and permeabilization kit, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and stained intracellularly with anti-IFNγ-APC and anti-MIP-1β-PE (BD Biosciences). Surface expression of CD107a and intracellular production of IFNγ and MIP1β by NK cells (CD16<sup>+</sup>/56<sup>+</sup>CD3<sup>-</sup>) were then analysed by flow cytometry. For each Fc-assay, a negative control (human purified IgG, Sigma) was included. Mean of signal in the negative controls wells was subtracted as background signal. The gating strategy for the Fc-mediated functions are illustrated in Supplementary Fig. 1, <http://links.lww.com/QAD/C201>.

### Fc-mediated polyfunctionality

Polyfunctionality is represented as the summed *z* scores for each child's Fc-mediated function. *z* scores were determined by subtracting the mean function of all children from an individual child's value and dividing this by the standard deviation.

### Analysis

Statistical analyses were performed in Graphpad Prism version 8.4.1 (GraphPad, Software, San Diego, California, USA). Mann-Whitney nonparametric tests were used to compare groups and associations determined by Spearman test. *P* less than 0.05 considered significant.

## Results

### Study population clinical characteristics

Children were categorized by age, as per the ART initiation WHO guidelines at the time of study [28] (Table 1). Five children had age data missing from the records, four were less than 24 months of age, 17 were between 25 and 59 months, and 24 were above 59 months of age. Forty-six percent (23 of 50) of the children were female (Table 1). Viral load data was available for 44 of 50 with a medium HIV RNA copies/ml of 36 644 [IQR, 1815–98565], median CD4<sup>+</sup> cell counts/µl blood was 912 [IQR, 608–1422], median percentage CD4<sup>+</sup> was 20.8 [IQR, 13.0–29.0], lymphocyte counts 4.0 [3.6–5.5] × 10<sup>3</sup> cells/µl, whereas the median haemoglobin levels was 10.0 [10–11] mg/dl for all the children.

### HIV-specific antibody neutralization activity

Neutralization breadth was determined for 44 children against a globally representative panel of 12 viruses

**Table 1. Clinical characteristics of the study population at the time of sampling.**

Clinical parameters		N or median [25th–75th percentile]
Age (years)	Missing	5
	<24 months	4
	25–59 months	17
	60 months	24
Gender	Female	23
	Male	27
% CD4 <sup>+</sup>	<24 months	22.4 [14.6–30.2]
	25–59 months	20.7 [15.4–30.9]
	60 months	20.8 [9.3–25.9]
	All children	20.8 [13.0–28.8]
CD4 <sup>+</sup> cell count (cells/μl)	<24 months	1979 [1201–2739]
	25–59 months	1034 [759–1580]
	60 months	715 [567–1052]
	All children	912 [608–1422]
Viral load (copies/ml)	<24 months	1314.5 [913.5–272.752]
	25–59 months	37075 [2687–123.335]
	60 months	36749 [7632–92.559]
	All children	36.644 [1815–98.565]
Lymphocyte count (10 <sup>3</sup> /μl)	<24 months	8.1 [6.4–12.5]
	25–59 months	5.0 [4.0–5.9]
	60 months	3.8 [3.4–4.3]
	All children	4.0 [3.6–5.5]
Hb conc. (mg/dl)	<24 months	10.0 [8–12]
	25–59 months	10.0 [9–11]
	60 months	10.5 [10–11]
	All children	10.0 [10–11]

Baseline characteristics for the children at enrolment into study. Children were divided into age brackets as per the WHO ART initiation guidelines at the time of study. Five children had age missing from the data records and age is indicated as missing. Lymphocyte counts are represented as counts per 10<sup>3</sup> cells per μl of blood. All children; total number of children included in the study. N, total number of children; Hb, haemoglobin.

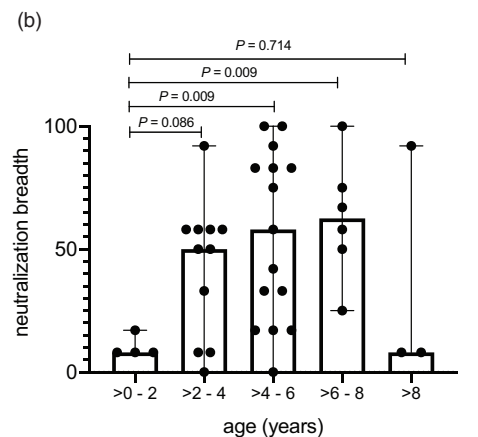
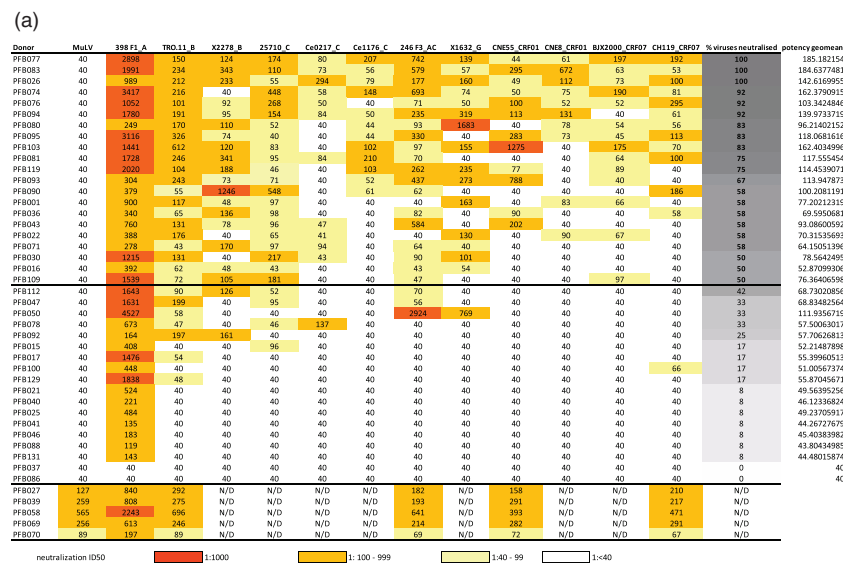
(6 children were excluded as EDTA plasma was not available, and heparin plasma results in high background in this assay) (Fig. 1a). Of the 44 plasma samples tested,

five showed activity against murine leukaemia virus (MuLV) envelope pseudotyped-viruses, a negative control, suggesting these plasmas contained antiretroviral drugs, despite self-reporting. These children were, therefore, excluded from all further analysis.

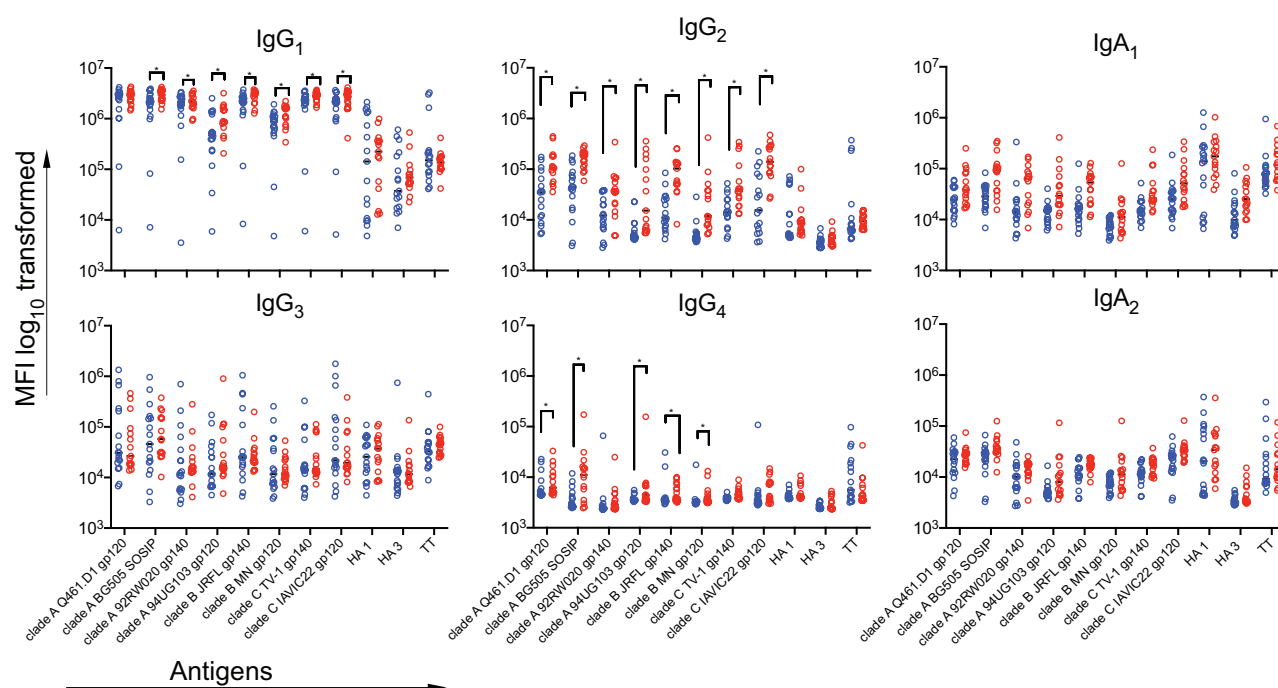
Virus neutralization ranged from 0 to 100% breadth against this panel, with potency highest against the tier 1 virus 398F1, and moderate titres against the rest, which are tier 2 viruses, more typical of circulating strains (Fig. 1a). Fifty-four percent of the children (21 of 39) neutralized more than half of the viruses tested and were defined as broad neutralizers in this study, including three children who neutralized all the viruses in this panel. Neutralization breadth was significantly higher in the older children, plateauing at about 4 years after infection (Fig. 1b) similar to studies of neutralization breadth in adults [32,33]. Plasma neutralization breadth and geometric mean titre were linearly associated (Spearman’s rho = 0.98, P < 0.0001) (Supplementary Fig. 2a, <http://links.lww.com/QAD/C202>), and therefore, neutralization breadth was used in all downstream association analyses.

**HIV-specific antibody levels and subtypes**

We next assessed HIV-specific antibody levels and isotype-specific binding to several HIV antigens. All children had IgG responses to the clade A BG505 gp120, as measured by ELISA. A large proportion of these were IgG<sub>1</sub> with antibody levels to the other IgG subclasses or IgA being lower (Supplementary Fig. 3a, <http://links.lww.com/QAD/C203>).



**Fig. 1. HIV-specific antibody neutralization function.** (a) Antibody neutralization was determined against 12 globally representative viruses using the TZM-bl neutralization assay. Neutralization titre is represented as the reciprocal of the plasma dilution required to inhibit 50% virus infection (ID<sub>50</sub>). Data shown as a heat map with titres greater than 1000 in red, 100–999 in orange, 41–99 in yellow and less than 40 in white. Titres of 1 : 40 or less indicate no neutralization. Neutralization breadth per sample; percentage of viruses neutralized. Potency; plasma dilution needed to achieve 50% viral infectivity and the geometric mean determined. (b) Neutralization breadth by age. Mann-Whitney test used and median and range indicated. P less than 0.05 considered significant.



**Fig. 2. Comparison of HIV-specific antibody levels in children with and without antibody neutralizing breadth.** Antigen-specific IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgA<sub>1</sub> and IgA<sub>2</sub> antibodies were quantified using an antigen-specific isotype multiplex assay. *x*-axis, antigens tested, *y*-axis log<sub>10</sub> transformed mean fluorescence intensity (MFI). Blue open circles indicate children with less than 50% neutralization breadth; red open circles indicate children with greater than 50% neutralization breadth. The median of antibody levels against each antigen is presented. Nonparametric Mann–Whitney test with *P* less than 0.05 being significant used for comparison. Each sample was analysed in technical duplicates. H1, A/California/07/2009; H3, A/Victoria/3/75; TT; inactivated Tetanus toxoid.

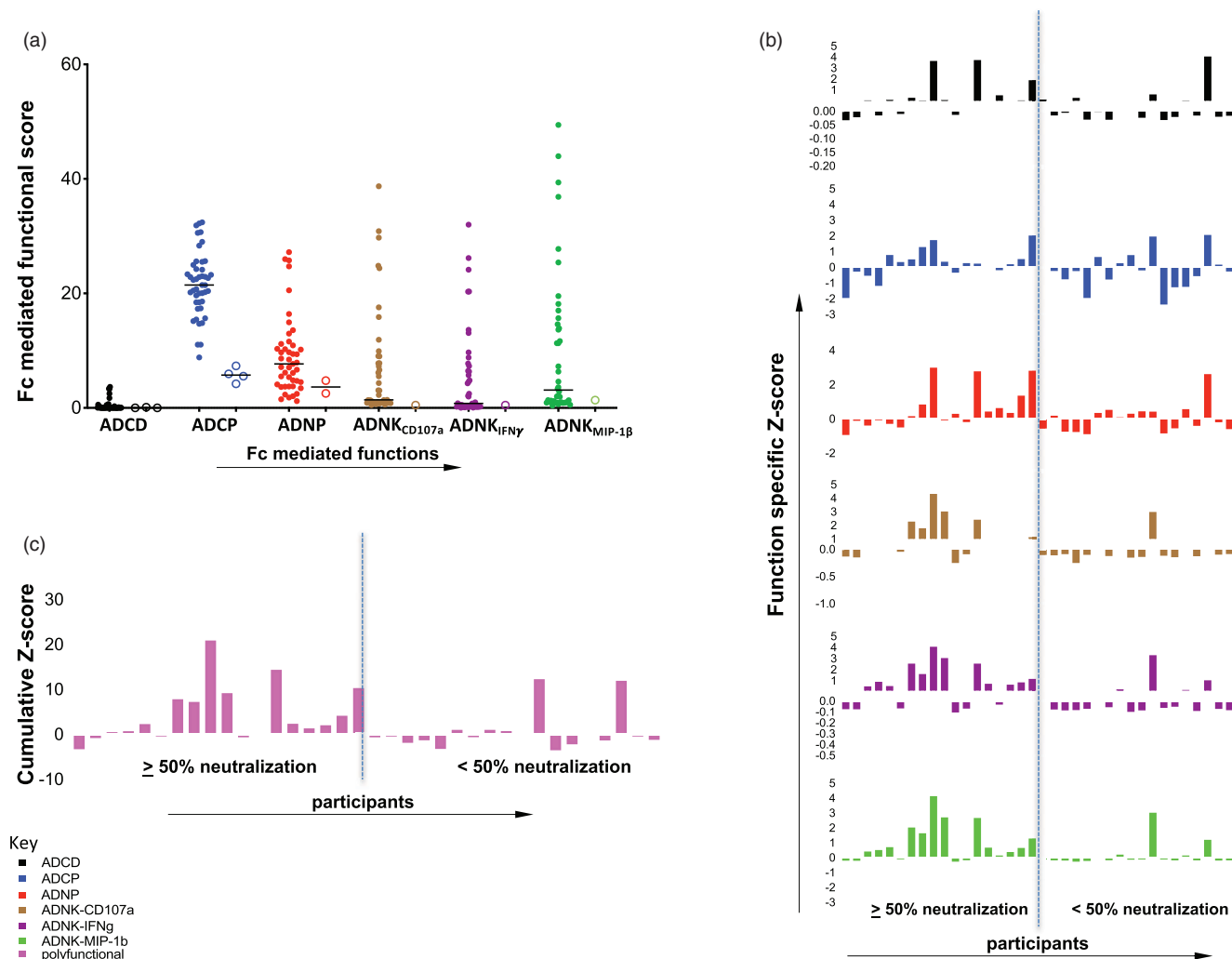
A similar pattern in antibody classes and isotype levels was observed using a multiplex microsphere bead assay incorporating a range of HIV antigens (Fig. 2). Antibody cross-reactivity to envelopes from clades B and C were observed and for some of the antigens, binding was at similar levels to the clade A antigen responses. Similar to the ELISA data, the predominant antibody subclass was IgG<sub>1</sub>. For IgG<sub>1</sub>, IgG<sub>2</sub> and IgG<sub>4</sub>, binding levels to several antigens were higher in children who developed neutralization breadth. This was HIV-specific as anti-tetanus toxoid and anti-HA influenza antibodies (Fig. 2) and total IgG were similar in both groups (Supplementary Fig. 3b, <http://links.lww.com/QAD/C203>).

### HIV-specific Fc-mediated antibody functions and polyfunctionality in children

Fc-mediated functions varied across the participants and by function. Complement deposition was detected in only 16% (8 of 50) of children, as expected from the low IgM titres and IgG<sub>3</sub> titres observed. A large proportion of the children's plasma, initiated phagocytosis either by a monocyte cell line THP-1 (ADCP) (100% of the children), or by neutrophil uptake (ADNP) (82% of the children) (Fig. 3a) with these two assays correlating (Spearman  $\rho = 0.58$ ,  $P < 0.0001$ ) confirming similar mechanisms (Supplementary Fig. 4a, <http://links.lww.com/QAD/C204>) [38]. The expression of CD107a (a

marker expressed upon natural killer cell degranulation) and IFN $\gamma$  and MIP-1 $\beta$ , two intracellular cytokines produced upon activation, were measured as proxies of antibody-dependent natural killer (ADNK) cell function. These were variable across samples, with ADNK<sub>CD107a</sub>, ADNK<sub>IFN $\gamma$</sub> , ADNK<sub>MIP-1 $\beta$</sub>  detected in 98, 58, and 60% of children, respectively. CD107a expression and IFN $\gamma$  and MIP-1 $\beta$  production were directly correlated as previously observed [21,39] (Supplementary Fig. 4b–d, <http://links.lww.com/QAD/C204>). Therefore, either of these markers could be used independently as a marker for natural killer cell function.

Z scores for each Fc function were used to compare the distribution of functions between neutralizers and nonneutralizers. A positive *z* score was more common in children who developed neutralization breadth suggesting the possibility of common determinants for function (Fig. 3b). To establish if these independent Fc-mediated functions were coordinated, we calculated a polyfunctionality score. A summed *z* score greater than 0 represented coordinated Fc-mediated functions (Fig. 3c). Thirty-six children with a polyfunctionality score had neutralization data available. Notably, 13 of 18 of the children who generated neutralization breadth showed coordinated Fc-mediated antibody functions compared with five of 18 of the children with poor neutralization



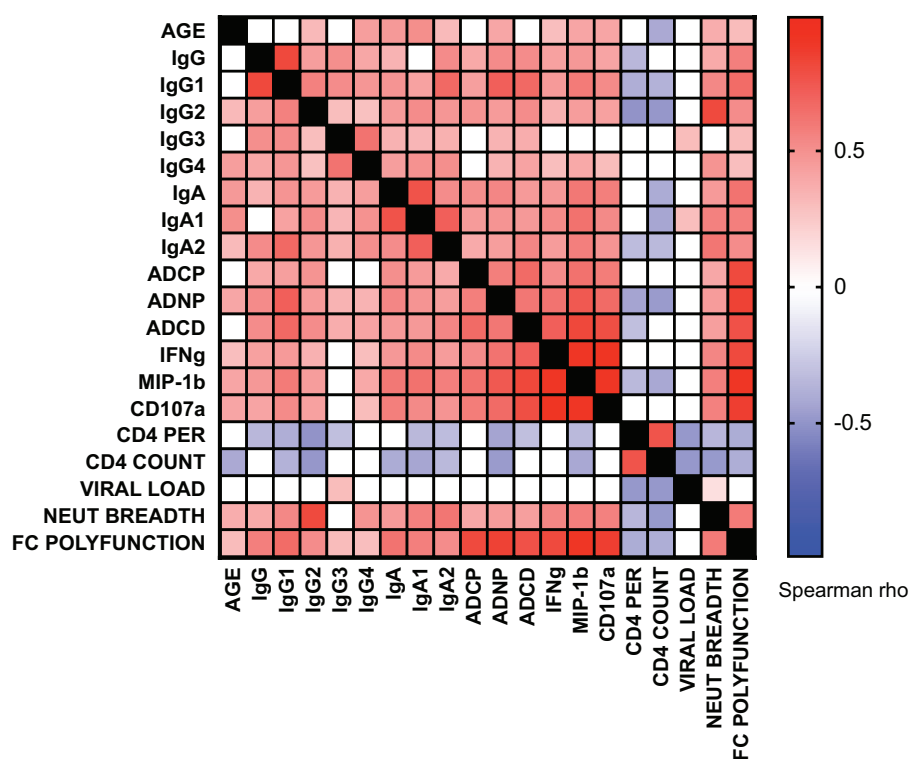
**Fig. 3. HIV-specific Fc-mediated antibody functions.** The ability of diluted children plasma to drive the uptake of gp120-coated fluorescent beads by THP-1 cells [antibody-dependent cellular phagocytosis (ADCP)], by neutrophils [antibody-dependent neutrophil phagocytosis (ADNP)], promote complement deposition [antibody-dependent complement deposition (ADCD)]; or activate NK cells [antibody-dependent natural killer (NK) cell degranulation (ADNK)] reflected by surface expression of CD107a and IFN $\gamma$  and MIP-1 $\beta$  production was evaluated. (a) Median functional score for the tested Fc-mediated functions. Median score shown, open circles; HIV-negative controls, closed circles; HIV-positive participants. (b) z scores for each child's Fc-mediated functions. (c) Polyfunctionality score (summed z scores of each Fc-mediated function for each child). Experiments were performed in duplicates.

breadth. Our data show that the development of neutralization breadth was associated with a more coordinated Fc-mediated function.

### Association of antibody titres and functions with clinical parameters

Given the range of Fc effector functions and antibody titres in this cohort, we sought to understand the association of these with clinical parameters, such as age, CD4<sup>+</sup> frequency (% of total PBMCs), CD4<sup>+</sup> counts and viral load. As HIV infection in this cohort was a result of vertical transmission from mother to child, the child's age reflects duration of HIV infection.

As the pattern of antibody levels was similar across the eight gp120 HIV antigens tested (Fig. 2), BG505 SOSIP responses, representative of the prevailing clade in the study region, were tested for their association with antibody function (Fig. 4). IgG antibody levels directly correlated with neutralization breadth (Spearman's  $\rho = 0.3954$ ,  $P = 0.0170$ ) and with ADCD, ADCP, ADNP, ADNK<sub>IFN $\gamma$</sub>  and ADNK<sub>MIP-1 $\beta$</sub>  secretion (Supplementary Table 1, <http://links.lww.com/QAD/C205>). Similarly, the coordinated Fc-mediated function represented by the polyfunctional score was associated with IgG titres (Spearman's  $\rho = 0.5769$ ,  $P = 0.0001$ ).



**Fig. 4. Heatmap representation of HIV-specific antibody function association with clinical outcomes, antibody levels and classes/subtypes: Spearman rho is shown where  $P$  less than 0.05, white boxes represent no significant association.** ADCD, antibody-dependent complement deposition; ADCP, antibody-dependent cellular phagocytosis; ADNK, antibody-dependent natural killer (NK) cell degranulation functions; ADNP, antibody-dependent neutrophil phagocytosis; NEUT BREADTH, neutralization breadth.

IgG subclasses IgG<sub>1</sub>, IgG<sub>2</sub> and IgG<sub>4</sub> were also directly associated with antibody neutralization breadth (Supplementary Table 1, <http://links.lww.com/QAD/C205>, Spearman's  $\rho = 0.5456$ ,  $P = 0.0012$ ; Spearman's  $\rho = 0.8114$ ,  $P = 0.0001$ ; Spearman's  $\rho = 0.4892$ ,  $P = 0.0045$ , respectively) but IgG<sub>3</sub> was not. Additionally, both IgG<sub>1</sub> and IgG<sub>2</sub> sub-classes showed strong associations with individual Fc-mediated functions, and with the overall polyfunctionality, whereas IgG<sub>3</sub> and IgG<sub>4</sub> were poorly associated. IgA antibody levels were also associated with neutralization breadth (Spearman's  $\rho = 0.4621$ ,  $P = 0.0046$ ) and this association was observed for both the IgA<sub>1</sub> and IgA<sub>2</sub> subclasses. Similarly, IgA antibody levels were correlated with all Fc-mediated functions tested, and a strong association with Fc-mediated polyfunctionality score was also observed (Spearman's  $\rho = 0.6313$ ,  $P = 0.0001$ ). Overall, higher antibody levels reflected better HIV-specific antibody functions.

As suggested in Fig. 1b, there was a direct association between age and neutralization breadth (Supplementary Table 1, <http://links.lww.com/QAD/C205>, Spearman's  $\rho = 0.3766$ ,  $P = 0.0258$ ). Some Fc-mediated functions, namely ADNP and ADNK, also showed an association with age, suggesting these antibody functions improved

over time. However, ADCP and ADCD were not associated with age, suggesting these Fc-mediated functions are generated early in infection and do not significantly improve over time.

Neutralization breadth was negatively associated with both CD4<sup>+</sup> cell counts and percentage as in previous studies [15] (Supplementary Table 1, <http://links.lww.com/QAD/C205>, Spearman's  $\rho = -0.4663$ ,  $P = 0.0054$ , Spearman's  $\rho = -0.3454$ ,  $P = 0.0490$ , respectively). However, Fc-mediated functions were less strongly associated with reduced CD4<sup>+</sup> cell counts and percentages. Although a consistent trend was observed, significance was only achieved for ADNP and ADNK<sub>MIP-1 $\beta$</sub>  expression (Spearman's  $\rho = -0.4582$ ,  $P = 0.0020$ ; Spearman's  $\rho = -0.4120$ ,  $P = 0.0074$ ) and for the polyfunctionality score (Spearman's  $\rho = -0.3821$ ,  $P = 0.0115$ ). HIV-specific antibody functions were, therefore, not associated with slowed clinical deterioration. There was no significant association between viremia and neutralization breadth (Spearman's  $\rho = 0.1509$ ,  $P = 0.3870$ ) or the different Fc-mediated antibody functions.

Taken together, these data suggest that neutralizers have greater, broader, and more functional humoral responses,

which are in some cases correlated with age and CD4<sup>+</sup> cell count, but independent of viral load.

## Discussion

Here, we measured HIV-specific antibody levels, neutralization breadth and Fc-mediated functions in chronically HIV-infected, ART-naïve children. Neutralization breadth developed in more than half of the children and correlated with Fc-mediated functions, suggesting shared determinants of enhanced antibody function exist. Our observations support children's ability to recognize and mount robust immune responses against HIV antigens, confirming the potential of this target population for HIV immunization strategies.

Emerging data suggests that a larger proportion of children than adults develop neutralization breadth [14,15,40]. In agreement with this, we observed that 54% of the children neutralized more than 50% of the viruses in the representative test panel. Extended viral exposure may have allowed for activation of multiple B-cell lineages, favouring the acquisition of neutralization breadth. Additionally, higher numbers of effector T-follicular helper cells in children [41] may provide enhanced help than in adults for B-cell differentiation.

In this cross-sectional study, viral load was not an independent predictor of neutralization breadth. It is likely that the quality of antibody function at the time of sampling was determined by earlier or accumulated events over the course of infection, such as early viremia, as has been previously demonstrated [33]. The modest association previously reported [15] further supports the existence of alternative drivers of breadth in addition to viraemia.

Antibody neutralization breadth correlated with total IgG, IgG<sub>2</sub> and IgA titres. Although IgG<sub>2</sub> responses were lower, as previously reported [42,43], a strong association with neutralization breadth was observed, similar to adults [26]. Low peripheral IgA antibody levels were observed, in agreement with expected dominance in the mucosal environment [44,45]. However, these were highly associated with neutralization. It is likely that multiple Fc-related mechanism are involved in viral control [46,47] and that a balance in subtype and class distribution is necessary for the generation of antibody neutralization breadth [47]. Further studies to understand this linkage are necessary.

Only a small proportion of children had plasma antibodies that could drive complement deposition (ADCD). The undetectable levels of IgM (data not shown) and low IgG<sub>3</sub> antibody levels, both good mediators of complement, may have contributed to low complement deposition. It

is possible that after years of infection, circulating HIV-specific antibodies in these children were largely switched to IgG subtype [45]. IgM, a first-line antibody may have been detected earlier in infection and not at this chronic phase [48]. However, in the few children where ADCD was detectable, there was a strong association between complement deposition and total IgG and IgA as previously observed [26,49] and with IgG<sub>1</sub>. Higher IgG levels may have resulted in more IgG-antigen complex formation. Similarly, ADCD showed strong association with neutralization breadth supporting previous reports [26]. A potential mechanism for this association is the binding of C3 to complement-receptor-2 on follicular dendritic cells, which may improve antigen presentation in the germinal centres leading to higher affinity maturation and improved antibody breadth [50,51].

Unlike ADCD, the other Fc-mediated functions were more common. All the children in this cohort mediated ADCP, and the phagocytic score was high for most children as has been observed in adults over the course of HIV infection [26]. In RV144 vaccinees, depletion of IgG<sub>3</sub> antibodies led to a significant loss of ADCP, although other subclasses were shown to be involved [52]. In our chronic infection cohort, few children had detectable levels of IgG<sub>3</sub>. Decreasing levels of IgG<sub>3</sub> with disease progression have been previously reported [45,53,54] and may reflect downstream antibody switching. It is likely that the ADCP antibody function we observed was driven by highly functional IgG<sub>1</sub> antibodies [55,56]. We further assayed phagocytosis via neutrophils (ADNP). Neutrophils are common and active in all sites of HIV infection. Phagocytosis by neutrophils has been reported to peak much faster than primary NK cell or monocyte-mediated responses suggesting its role in early viral control [57]. The induction of phagocytosis via distinct innate Fc effector cell types would be beneficial in mounting efficient viral clearance mechanisms.

We then measured multiple effector functions of activated NK cells including degranulation and the release of IFN $\gamma$  and MIP-1 $\beta$  cytokines as proxies of antibody-dependent cell death. Previous comparisons have shown association between ADCC to CD107a degranulation [58] and in our study, there was positive correlation between CD107a, IFN $\gamma$  and MIP-1 $\beta$  as previously observed [21,39] suggesting that either of these markers could be used as a proxy for ADCC. IgG<sub>1</sub> mediates antiviral functions by binding to FcR, mediating ADCC of infected cells [59] and may have been critical in mediating the effector functions.

Previous studies suggest that multiple Fc-mediated functions provide superior viral clearance mechanisms by recruiting multiple players of innate immunity [21,26,37]. Here, 23 out of 50 (46%) children generated coordinated Fc-mediated functions. Importantly, a larger



proportion of children who showed coordinated Fc-mediated effector functions also generated neutralization breadth, suggesting that antibodies function synergistically, consistent with emerging data of joint regulation of Fc-mediated and Fab-mediated antibody functions [26,60,61]. The identification of signatures for germinal centre activities that link these processes may be exploited in vaccine design to drive desired multifunctional antibody responses.

One limitation of the study is that IgG was not purified prior to performing the assays, which would have enabled a more realistic comparison of antibody 'quality'. The cross-sectional study design also limited the association with earlier events, such as viral set point and changes over time or with clinical outcome.

Our findings show that children mount potent antibody responses to HIV antigens and that a large proportion develop neutralization breadth and Fc-mediated antibody functions. HIV vaccination strategies targeting children may, therefore, prove more likely to yield desired responses. The use of a coordinated multifunctional systems approach in key populations, such as paediatric donors, thus provides a comprehensive characterization of desirable antibody functions.

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## Conflicts of interest

There are no conflicts of interest.

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