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Comparison of Antibody-Dependent Cell-Mediated Cytotoxicity and Virus Neutralization by HIV-1 Env-Specific Monoclonal Antibodies

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ABSTRACT

Although antibodies to the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein have been studied extensively for their ability to block viral infectivity, little data are currently available on nonneutralizing functions of these antibodies, such as their ability to eliminate virus-infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC). HIV-1 Env-specific antibodies of diverse specificities, including potent broadly neutralizing and nonneutralizing antibodies, were therefore tested for ADCC against cells infected with a lab-adapted HIV-1 isolate (HIV-1_{NL4-3}), a primary HIV-1 isolate (HIV-1_{JR-FL}), and a simian-human immunodeficiency virus (SHIV) adapted for pathogenic infection of rhesus macaques ($SHIV_{AD8-EO}$). In accordance with the sensitivity of these viruses to neutralization, HIV-1_{NL4-3}-infected cells were considerably more sensitive to ADCC, both in terms of the number of antibodies and magnitude of responses, than cells infected with HIV-1_{JR-FL} or $SHIV_{AD8-EO}$. ADCC activity generally correlated with antibody binding to Env on the surfaces of virus-infected cells and with viral neutralization; however, neutralization was not always predictive of ADCC, as instances of ADCC in the absence of detectable neutralization, and vice versa, were observed. These results reveal incomplete overlap in the specificities of antibodies that mediate these antiviral activities and provide insights into the relationship between ADCC and neutralization important for the development of antibody-based vaccines and therapies for combating HIV-1 infection.

IMPORTANCE

This study provides fundamental insights into the relationship between antibody-dependent cell-mediated cytotoxicity (ADCC) and virus neutralization that may help to guide the development of antibody-based vaccines and immunotherapies for the prevention and treatment of HIV-1 infection.

The recent isolation of a new generation of monoclonal antibodies with remarkably potent and broad neutralizing activity against diverse human immunodeficiency virus type 1 (HIV-1) isolates has renewed interest in the use of antibodies to treat HIV-1 infection (1, 2). Passive transfer experiments in animal models have shown that many of these antibodies can protect against HIV-1 or simian-human immunodeficiency virus (SHIV) challenge (3, 4), and in some cases, they are able to suppress virus replication to undetectable levels when administered during chronic infection (5–7). While the ability to block viral infection is a defining property of neutralizing antibodies, nonneutralizing effector functions may also contribute to antiviral responses. The IgG constant (Fc) domain can recruit cellular mediators of antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis through interactions with Fcγ receptors (FcγRs) or initiate complement-mediated lysis by binding to soluble factors in plasma.

Studies of nonhuman primates and mice support a role for FcγR-dependent functions of antibodies in protection against immunodeficiency virus infection. Passive transfer experiments with Fc variants of an HIV-specific broadly neutralizing antibody (bNAb) revealed that protection of rhesus macaques against pathogenic SHIV challenge is dependent in part on FcγR interactions, but not on complement fixation (8, 9). The preferential engagement of activating, but not inhibitory, FcγRs was also shown to contribute to the clearance of cell-free virus by antibodies in murine models (10), and FcγR-mediated functions of bNAbs interfered with the establishment of persistent HIV-1 reservoirs in humanized mice (11). Thus, the therapeutic potential of HIV-1-specific antibodies may be significantly enhanced by optimizing FcγR-dependent antiviral activities.

Emerging evidence suggests that antibodies capable of engaging FcγRIIIa on NK cells to direct the lysis of virus-infected cells may be especially important for containing or preventing HIV-1 infection (12, 13). ADCC responses are detectable in plasma

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shortly after the resolution of acute viremia and correlate inversely with disease progression (14–20). Greater ADCC responses have also been observed in individuals who exhibit elite control of HIV-1 in the absence of antiretroviral therapy (21, 22). In the setting of mother-to-child transmission, higher ADCC activity in breast milk is associated with a lower risk of virus transmission by breastfeeding, and passively acquired ADCC correlates with reduced infant mortality (23, 24). ADCC may also have contributed to the modest protection observed in the RV144 trial as suggested by exploratory analyses revealing an association between ADCC and reduced risk of infection among vaccinated subjects with low IgA titers (25). Although passive transfer of a nonfucosylated bNAb with increased affinity for FcγRIIIa did not enhance the protection of macaques against pathogenic SHIV challenge relative to the fucosylated antibody (26), several studies of nonhuman primates have also revealed correlations between vaccine-induced ADCC and complete protection or reduced postchallenge viral loads (27–31).

While these studies suggest that ADCC, and possibly other FcγR-dependent functions, contribute to the antiviral activity of HIV-1-specific antibodies, the properties of antibodies that mediate ADCC are not well defined. We therefore tested monoclonal antibodies to diverse epitopes of the HIV-1 envelope glycoprotein, including potent bNAbs and nonneutralizing antibodies, for their ability to direct NK cell lysis of cells infected with primary versus lab-adapted HIV-1 and SHIV isolates. These antibodies were also tested for binding to Env on the surfaces of virus-infected cells and for neutralization of viral infectivity. Our results show that although ADCC generally correlates with Env binding and neutralization, there are cases where these functions do not correspond, revealing differences in epitopes exposed on virions versus infected cells that differentiate these antiviral activities.

MATERIALS AND METHODS

Virus production. Virus stocks were produced by transfection of proviral DNA into HEK 293T cells using GenJet transfection reagent (SignaGen). Culture supernatants were collected 48 and 72 h posttransfection, cell debris was removed by centrifugation, and aliquots of virus-containing supernatant were stored at −80°C. Virus concentrations were determined by anti-p24 or anti-p27 enzyme-linked immunosorbent assay (ELISA). $SHIV_{AD8\text{-}EO}$ was provided by Malcolm Martin.

Antibodies. 2F5 and 4E10 were obtained from Polymun Scientific, and X5 and 17b were obtained from Strategic Biosolutions. b12, b6, and DEN3 were stably expressed in CHO-K1 cells. VRC01, PGV04, 2G12, PG9, PG16, PGT121, PGT126, and 10E8 were transiently expressed using the FreeStyle 293 Expression System (Invitrogen) as previously described (32, 33). A32, C11, 3BNC117, and 10-1074 were transiently expressed in HEK 293T or HEK 293-6E cells (5, 34, 35). 98-6, 126-7, 240D, and F240 were derived from Epstein-Barr virus (EBV)-transformed peripheral blood mononuclear cells (PBMCs) from HIV-1-positive ($HIV\text{-}1^+$) donors fused with a human-mouse heteromyeloma cell line according to established methods (36–40). All antibodies were purified from cell-free supernatant using protein A or protein G affinity chromatography. Antibody concentrations were determined by absorbance at 280 nm and a mass extinction coefficient of 13.7 for a 1% (10-mg/ml) IgG solution (Nanodrop; Thermo Scientific) or by anti-human IgG ELISA using isotype-matched control immunoglobulins.

ADCC assay. ADCC activity was measured as previously described (31, 41). CEM.NKR-$_{CCR5}$-sLTR-Luc target cells, which express luciferase (Luc) under the control of a Tat-inducible promoter, were infected by spinoculation in the presence of 40 μg/ml Polybrene. At 4 days postinfection, infected cells were incubated with monoclonal antibodies and an NK cell line expressing human CD16 for 8 h. The dose-dependent loss of Luc activity was measured as an indication of antibody-mediated killing of virus-infected cells. Infected target cells incubated with NK cells in the absence of antibody were used to measure maximal Luc activity, and uninfected target cells cultured with NK cells were used to determine background Luc activity. Percent relative light units (RLU) were used to determine partial area under the ADCC curve (pAUC) values and antibody concentrations required for half-maximal killing (50% ADCC), as previously described (25, 41). Differences between $\log_{10}$-transformed percent RLU values and 100% RLU, indicating no activity, were calculated. pAUC values were determined by multiplying the sum of these differences at the four highest antibody concentrations tested by the $\log_{10}$-transformed dilution factor of 2, yielding an area. Standard deviations (SD) of individual measurements were propagated to yield the SD of the pAUC.

Neutralization assay. Neutralization of viral infectivity was measured using a TZM-bl reporter cell assay, according to standard methods (42, 43). In a flat-bottom 96-well plate, 5,000 cells per well were seeded the day before the neutralization assay. Either 4 ng p24 ($HIV\text{-}1_{NL4\text{-}3}$), 10 ng p24 ($HIV\text{-}1_{JR\text{-}FL}$), or 20 ng p27 ($SHIV_{AD8\text{-}EO}$) of virus per well was incubated with serial dilutions of monoclonal antibody for 1 h at 37°C before being added to the reporter cells. After 3 days, luciferase activity in cell lysates was measured, and virus neutralization was calculated from reductions in RLU relative to cells incubated with virus but no antibody. Uninfected cells were measured to account for background luciferase activity. Partial area under the neutralization curve (pAUC) values and antibody concentrations for 50% neutralization (IC_{50}) were calculated by using the same methods as for the ADCC assay.

Flow cytometry. Surface envelope staining was performed 3 days postinfection as previously described (44, 45). Antibody binding to Env was detected using 5 μg/ml of monoclonal antibody followed by anti-human IgG $F(ab')_2$ (phycoerythrin [PE]; polyclonal). Cells were surface stained for CD45 (peridinin chlorophyll protein [PerCP]; clone 2D1) and CD4 (Alexa Fluor 700; clone RPA-T4), then permeabilized, and stained for intracellular Gag (fluorescein isothiocyanate [FITC]; clone FH190-1-1 for HIV-1; clone 55-2F12 for SHIV). Nonviable cells were excluded using LIVE/DEAD fixable dead cell aqua stain (Invitrogen), and data were collected using a SORP BD LSR-II flow cytometer (Becton Dickinson). After gating on viable $CD45^+$ $CD4^{low}$ Gag^+ cells, the geometric mean fluorescence intensity (gMFI) of Env staining was calculated using FlowJo, version 9.7.7 (Tree Star, Inc.).

Statistical analysis. All statistical analysis was done using Prism version 6.0g (GraphPad Software, Inc.). Correlations were determined by calculating Pearson product-moment correlation coefficients. Significance levels of ADCC activity and neutralization were calculated by comparing pAUC values of samples to negative-control values by two-way analysis of variance (ANOVA) with a Dunnett correction for multiple comparisons. Negative controls for ADCC assays were pAUC values of the same antibody against $SIV_{mac}239$-infected cells. For neutralization data, comparisons were drawn to the hypothetical pAUC of a negative sample with percent RLU of 100.

RESULTS

ADCC activity of HIV-1 Env-specific monoclonal antibodies. Monoclonal antibodies targeting diverse epitopes of the HIV-1 envelope glycoprotein were tested for ADCC against target cells infected with $HIV\text{-}1_{NL4\text{-}3}$ and $HIV\text{-}1_{JR\text{-}FL}$, which represent lab-adapted and primary HIV-1 isolates, respectively, with tier 1 versus tier 2 sensitivity to neutralizing antibodies, $SHIV_{AD8\text{-}EO}$, which is a chemokine (C-C motif) receptor 5 (CCR5)-tropic simian-human immunodeficiency virus isolate adapted for pathogenic infection of rhesus macaques (46–48), and $SIV_{mac}239$ as a control for nonspecific killing. The antibodies (all IgG1) included bNAbs to the CD4 binding site (CD4bs) (49–54), glycan and proteogly-

TABLE 1 Comparison of pAUC values for ADCC and virus neutralization[a]

Binding site	Antibody	HIV-1 NL4-3		HIV-1 JR-FL		SHIV AD8-EO	
		ADCC	Neut	ADCC	Neut	ADCC	Neut
CD4bs	b12	1.4 ±0.2	> 2.4	0.58 ±0.03	> 2.4	n.s.	2.2 ±0.1
	b6	1.3 ±0.1	1.8 ±0.1	n.s.	n.s.	n.s.	n.s.
	VRC01	1.2 ±0.1	> 2.4	0.43 ±0.02	> 2.4	n.s.	> 2.4
	PGV04	0.73 ±0.03	> 2.4	0.56 ±0.02	> 2.4	0.33 ±0.01	> 2.4
	3BNC117	1.8 ±0.1	> 2.4	0.79 ±0.03	> 2.4	0.17 ±0.01	> 2.4
V2 apex proteoglycan	PG9	0.42 ±0.02	1.0 ±0.0	0.28 ±0.01	0.27 ±0.01	n.s.	n.s.
	PG16	0.42 ±0.02	1.1 ±0.0	0.09 ±0.01	0.24 ±0.01	n.s.	n.s.
V3 proteoglycan	PGT126	0.53 ±0.04	0.28 ±0.01	0.96 ±0.05	> 2.4	0.28 ±0.02	> 2.4
	PGT121	0.08 ±0.02	n.s.	0.76 ±0.05	> 2.4	0.61 ±0.02	> 2.4
	10-1074	0.71 ±0.02	0.22 ±0.02	0.63 ±0.02	> 2.4	0.20 ±0.02	> 2.4
oligomannose	2G12	0.08 ±0.02	1.8 ±0.0	n.s.	2.1 ±0.1	n.s.	1.1 ±0.1
MPER	2F5	n.s.	1.9 ±0.0	n.s.	0.87 ±0.04	n.s.	0.93 ±0.07
	4E10	n.s.	0.98 ±0.03	n.s.	0.65 ±0.02	n.s.	0.58 ±0.03
	10E8	0.18 ±0.01	> 2.4	n.s.	2.2 ±0.1	n.s.	2.1 ±0.1
CD4i	17b	0.47 ±0.02	1.9 ±0.0	n.s.	0.13 ±0.02	n.s.	0.24 ±0.03
	X5	0.71 ±0.03	1.4 ±0.0	n.s.	n.s.	n.s.	n.s.
C1/C5	A32	0.17 ±0.02	n.s.	n.s.	n.s.	n.s.	n.s.
	C11	0.20 ±0.02	n.s.	n.s.	n.s.	n.s.	n.s.
cluster I	240D	0.77 ±0.04	n.s.	n.s.	n.s.	n.s.	n.s.
	F240	0.33 ±0.03	n.s.	n.s.	n.s.	n.s.	n.s.
cluster II	98-6	0.11 ±0.03	n.s.	n.s.	n.s.	n.s.	n.s.
	126-7	0.15 ±0.02	n.s.	n.s.	n.s.	n.s.	n.s.

[a] Percent RLU values at the four highest antibody concentrations tested were used to calculate partial area under the curve (pAUC) values as previously described (25, 41). Standard deviations were calculated from triplicate measurements. Red indicates potent ADCC or neutralization (Neut) (top tertile), yellow indicates intermediate activity, green indicates weak activity (bottom tertile), and blue indicates a lack of significant antiviral activity ($P > 0.01$). The tertiles for ADCC activity and neutralization were calculated separately from the respective values against HIV-1_{NL4-3}. n.s., not significant.

can epitopes in gp120 (33, 55–60), and the membrane-proximal external region (MPER) of gp41 (55, 61–63) and nonneutralizing antibodies to CD4-inducible (CD4i) epitopes in gp120 (53, 64–67) and cluster I and cluster II epitopes of gp41 (39, 40, 68–72). ADCC was assessed using an assay designed to measure the lysis of virus-infected cells expressing native conformations of Env, and antibody concentrations for half-maximal lysis (50% ADCC) and partial area under the curve (pAUC) values were calculated as previously described (25, 41).

HIV-1_{NL4-3}-infected cells exhibited the greatest susceptibility to ADCC. Of the 22 antibodies tested, 20 mediated significant lysis of cells infected with this virus (Table 1 and Fig. 1). These antibodies included most of the bNAbs (except 2F5 and 4E10), antibodies to CD4i epitopes in the coreceptor binding site (17b and X5), as well as nonneutralizing antibodies to the gp120 inner domain (A32 and C11) and nonneutralizing antibodies to gp41 (F240, 240D, 98-6, and 126-7). In most cases, HIV-1_{NL4-3}-infected cells were also susceptible to ADCC at much lower antibody concentrations than cells infected with HIV-1_{JR-FL} or $SHIV_{AD8-EO}$ (Fig. 1). Thus, consistent with the well-documented sensitivity of the lab-adapted isolate HIV-1_{NL4-3} to neutralizing antibodies (73–75), cells infected with HIV-1_{NL4-3} were highly susceptible to ADCC.

In contrast, cells infected with HIV-1_{JR-FL} and $SHIV_{AD8-EO}$ were susceptible to lysis only by bNAbs. With the exception of the oligomannose-specific antibody 2G12 and the MPER-specific antibodies 2F5, 4E10, and 10E8, HIV-1_{JR-FL}-infected cells were sensitive to all of the bNAbs (Table 1 and Fig. 1); however, $SHIV_{AD8-EO}$-infected cells were resistant to all but a handful of antibodies. ADCC was detected against $SHIV_{AD8-EO}$-infected cells for PGV04, 3BNC117, PGT126, PGT121, and 10-1074, but only PGV04 and PGT121 mediated potent killing at 50% ADCC concentrations of less than 100 μg/ml (58 μg/ml and 0.67 μg/ml, respectively) (Table 2). ADCC activity was also measured at lower antibody concentrations for HIV-1_{JR-FL}-infected cells than $SHIV_{AD8-EO}$-infected cells (Fig. 1). These observations indicate that $SHIV_{AD8-EO}$-infected cells are less sensitive to recognition by most HIV-1-specific antibodies, perhaps as a consequence of extensive adaptation of this virus for replication in rhesus macaques, as reflected by changes in the neutralization profile of $SHIV_{AD8-EO}$ relative to the parental HIV-1_{AD8} strain (76). In comparison to HIV-1_{NL4-3}, the greater resistance of HIV-1_{JR-FL}- and $SHIV_{AD8-EO}$-infected cells to

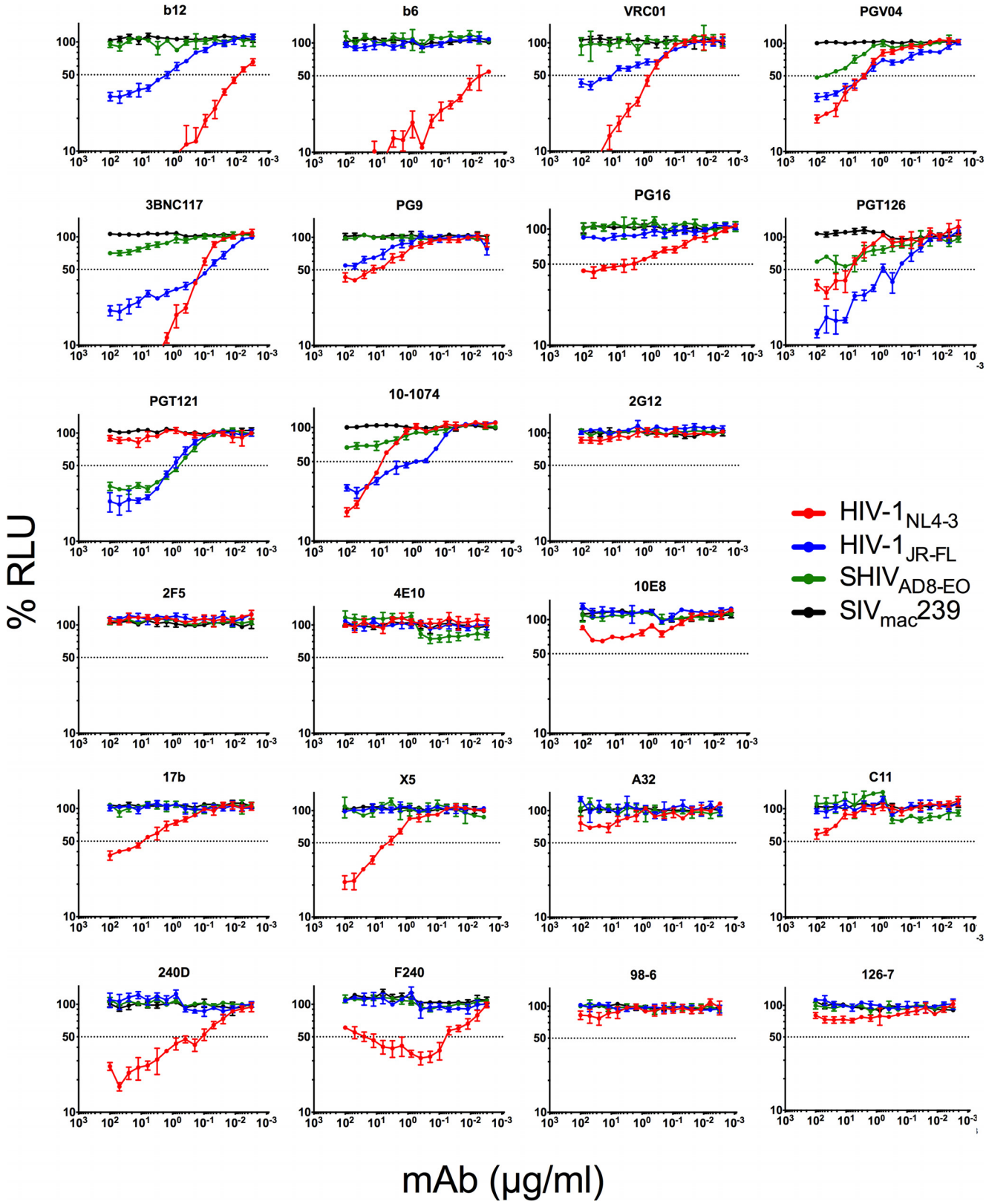

FIG 1 ADCC activity of HIV-1 Env-specific monoclonal antibodies. CEM.NKR-$_{CCR5}$-sLTR-Luc cells infected with HIV-1$_{NL4-3}$, HIV-1$_{JR-FL}$, SHIV$_{AD8-EO}$, or SIV$_{mac}$239 were incubated with an NK cell line expressing human CD16 at a 10:1 effector-to-target ratio in the presence of the indicated concentrations of monoclonal antibodies (mAbs). ADCC responses were measured as the dose-dependent loss of luciferase activity in relative light units (RLU) after an 8-h incubation in comparison to control wells containing NK cells and either infected (maximal) or uninfected (background) CEM.NKR-$_{CCR5}$-sLTR-Luc cells in the absence of antibody. Values are the means ± standard deviations (error bars) for triplicate wells, and the dotted line indicates half-maximal lysis of infected cells.

TABLE 2 Antibody concentrations for 50% ADCC and 50% neutralization[a]

Binding site	Antibody	HIV-1 NL4-3		HIV-1 JR-FL		SHIV AD8-EO	
		50% ADCC	IC50	50% ADCC	IC50	50% ADCC	IC50
CD4bs	b12	0.009 ±0.001	0.13 ±0.01	1.7 ±0.6	0.024 ±0.002	> 100	0.79 ±0.08
	b6	0.006 ±0.004	0.50 ±0.07	> 100	> 100	> 100	> 100
	VRC01	0.68 ±0.08	0.18 ±0.02	11 ±1	0.030 ±0.003	> 100	0.20 ±0.02
	PGV04	3.5 ±0.7	0.049 ±0.007	3.1 ±0.7	0.078 ±0.012	58 ±4	0.10 ±0.02
	3BNC117	0.14 ±0.01	0.007 ±0.002	0.080 ±0.007	< 0.003	> 100	0.012 ±0.001
V2 apex proteoglycan	PG9	14 ±7	0.60 ±0.04	> 100	> 100	> 100	> 100
	PG16	6.9 ±4.1	0.039 ±0.002	> 100	> 100	> 100	> 100
V3 proteoglycan	PGT126	9.8 ±2.1	78 ±12	0.71 ±0.38	0.030 ±0.004	> 100	0.021 ±0.001
	PGT121	> 100	> 100	1.0 ±0.2	0.039 ±0.005	0.67 ±0.06	0.018 ±0.009
	10-1074	9.3 ±0.2	87 ±11	1.7 ±1.5	0.034 ±0.000	> 100	0.022 ±0.002
oligomannose	2G12	> 100	0.66 ±0.12	> 100	0.63 ±0.02	> 100	2.6 ±0.1
MPER	2F5	> 100	2.9 ±0.4	> 100	10 ±4	> 100	7.9 ±2.9
	4E10	> 100	11 ±0	> 100	14 ±2	> 100	9.2 ±1.4
	10E8	> 100	0.016 ±0.005	> 100	0.46 ±0.08	> 100	0.22 ±0.05
CD4i	17b	9.5 ±0.6	0.16 ±0.02	> 100	> 100	> 100	91 ±15
	X5	3.9 ±1.2	2.1 ±0.2	> 100	> 100	> 100	> 100
C1/C5	A32	> 100	> 100	> 100	> 100	> 100	> 100
	C11	> 100	> 100	> 100	> 100	> 100	> 100
cluster I	240D	0.12 ±0.03	> 100	> 100	> 100	> 100	> 100
	F240	0.066 ±0.004	> 100	> 100	> 100	> 100	> 100
cluster II	98-6	> 100	> 100	> 100	> 100	> 100	> 100
	126-7	> 100	> 100	> 100	> 100	> 100	> 100

[a] Antibody concentrations (μg/ml) for half-maximal ADCC (50% ADCC) and virus neutralization (IC50) were calculated as previously described (41). Standard deviations were calculated from triplicate neutralization curves. Red indicates potent ADCC or neutralization (top tertile), yellow indicates intermediate activity, green indicates weak activity (bottom tertile), and blue indicates less than 50% activity at 100 μg/ml. The tertiles for ADCC activity and neutralization were calculated separately from the respective values against HIV-1_{NL4-3}.

ADCC is also consistent with the resistance of these primary isolates to neutralizing antibodies.

ADCC activity correlates with binding to Env on the surfaces of virus-infected cells. Antibody binding to virus-infected cells is a prerequisite for ADCC. Cells infected with HIV-1_{NL4-3}, HIV-1_{JR-FL}, and $SHIV_{AD8-EO}$ were therefore stained with each of the HIV-1 Env-specific monoclonal antibodies and analyzed by flow cytometry to determine the extent to which binding correlates with susceptibility to ADCC (Fig. 2). The geometric mean fluorescence intensities of Env staining (Table 3) were compared by nonparametric Spearman correlation to partial area under the curve values for ADCC (Fig. 3), which capture responses for antibodies that did not achieve 50% ADCC at concentrations less than 100 μg/ml.

Antibody binding correlated with ADCC for each of the three viruses tested. Whereas binding was highly predictive of ADCC for HIV-1_{JR-FL} ($P < 0.0001$), less robust, but nevertheless significant, associations were also observed for HIV-1_{NL4-3} ($P = 0.0104$) and $SHIV_{AD8-EO}$ ($P = 0.0422$) (Fig. 3). The relationship between binding and ADCC for HIV-1_{NL4-3}-infected cells reflects greater variability in these measurements and instances of antibody binding in the absence of detectable ADCC. For instance, 2F5 and 4E10 stained cells infected with HIV-1_{NL4-3} (Fig. 2), but they did not mediate cell lysis (Fig. 1). The reason for this discrepancy is unclear, but it may be related to the limited accessibility of these antibodies for engagement by FcγRs on NK cells when bound to virus-infected cells due to their specificity for an epitope consisting of phospholipids and sequences exposed at the base of gp41 (77–80). For $SHIV_{AD8-EO}$-infected cells, there was almost complete correspondence between antibody binding and susceptibility to ADCC (Fig. 1 and 2); however, the significance of this relationship was limited in comparison to HIV-1_{JR-FL} by lower responses that were detectable for a smaller subset of antibodies.

Correlation of ADCC activity with virus neutralization. The antiviral activity of HIV-1-specific antibodies is typically defined by their ability to neutralize viral infectivity. Each of the HIV-1 Env-specific monoclonal antibodies was therefore tested for neutralization of HIV-1_{NL4-3}, HIV-1_{JR-FL}, and $SHIV_{AD8-EO}$ to investigate the relationship between their ability to block viral infectivity and to mediate NK cell lysis of virus-infected cells. Antibody concentrations for 50% neutralization (IC_{50}) (Table 2) were calculated from neutralization curves (Fig. 4), and corresponded well to previously published data (33, 50, 51, 59, 63, 81–84). pAUC values for neutralization were also determined (Table 1) and compared to pAUC values for ADCC by nonparametric Spearman correlation (Fig. 5).

Neutralization correlated with ADCC for all three viruses. This relationship was strongest for HIV-1_{JR-FL} ($P = 0.0002$), followed by $SHIV_{AD8-EO}$ ($P = 0.0010$) and HIV-1_{NL4-3} ($P = 0.0302$) (Fig. 5). Several instances of neutralization in the absence of detectable ADCC were observed, and in most cases, antibodies that directed ADCC against cells infected with a particular isolate also neutralized that virus (Tables 1 and 2). Indeed, all of the antibodies with

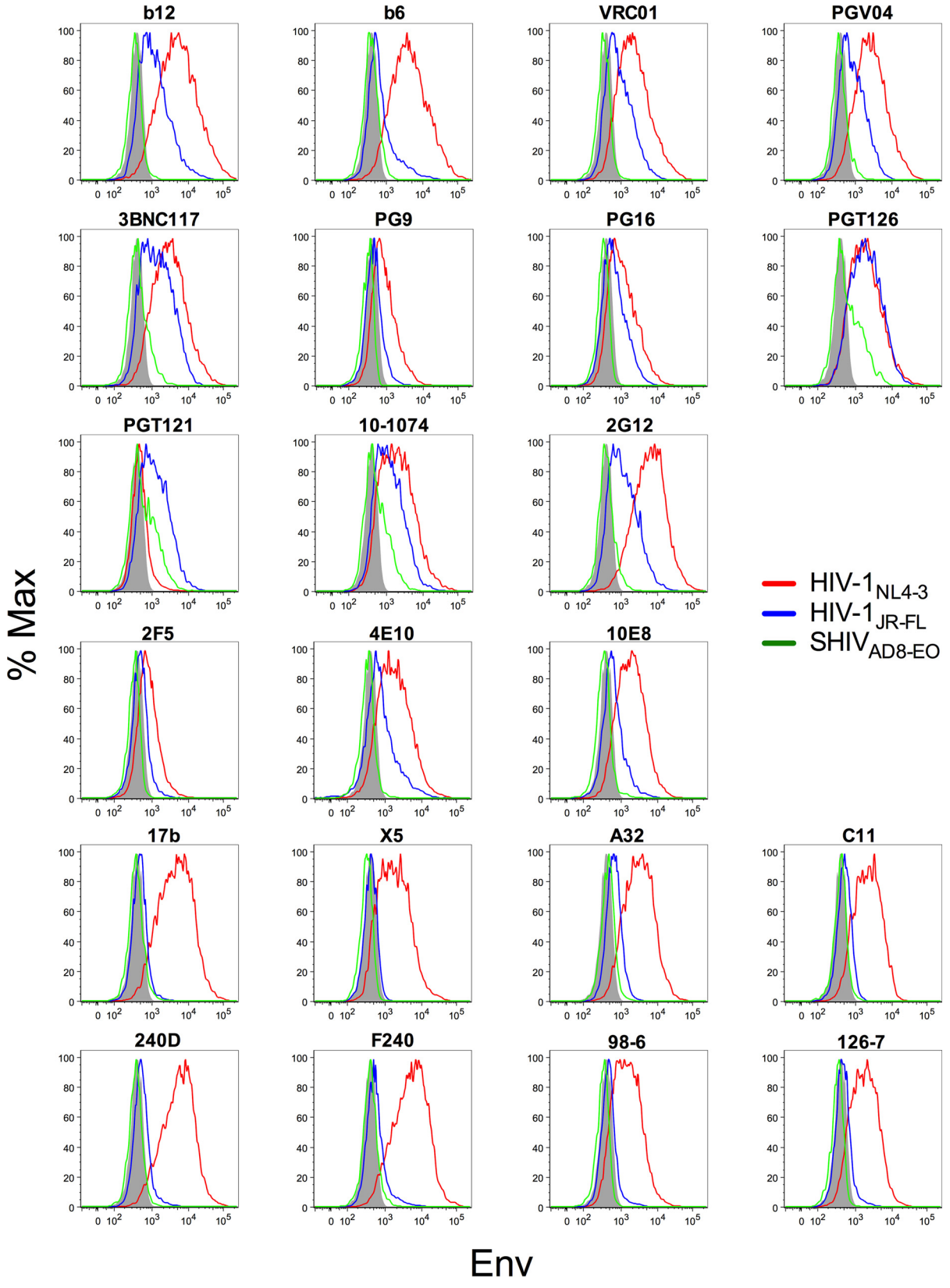

FIG 2 Antibody binding to Env on the surfaces of virus-infected cells. CEM.NKR-$_{CCR5}$-sLTR-Luc cells infected with HIV-1$_{NL4-3}$, HIV-1$_{JR-FL}$, or SHIV$_{AD8-EO}$ were stained with HIV-1 Env-specific antibodies followed by a PE-conjugated anti-human IgG F(ab′)$_2$. The cells were also stained for surface expression of CD45 and CD4, intracellular expression of the viral Gag protein, and with a viability dye. The histograms show Env staining on virus-infected (Gag$^+$ CD4^{low}) cells of the viable CD45$^+$ population. The shaded area indicates nonspecific staining with the DEN3 control antibody. Max, maximum.

TABLE 3 Env staining on the surface of virus-infected cells[a]

Antibody	gMFI of Env staining		
	HIV-1_{NL4-3}	HIV-1_{JR-FL}	$SHIV_{AD8-EO}$
DEN3	391	361	311
b12	5,370	1,179	355
b6	4,391	719	396
VRC01	2,289	901	351
PGV04	2,744	851	389
3BNC117	3,041	1,358	437
PG9	872	466	313
PG16	1,109	672	335
PGT126	1,882	1,930	620
PGT121	483	1,193	599
10-1074	1,951	1,191	540
2G12	6,167	1,148	411
2F5	800	482	333
4E10	1,768	756	341
10E8	1,836	619	349
17b	4,451	473	382
X5	1,743	412	322
A32	3,073	642	446
C11	2,108	504	396
240D	5,205	533	373
F240	4,996	560	402
98-6	1,438	473	327
126-7	1,740	471	331

[a] CEM.NKR-$_{CCR5}$-sLTR-Luc cells infected with HIV-1_{NL4-3}, HIV-1_{JR-FL}, or $SHIV_{AD8-EO}$ were stained with HIV-1 Env-specific antibodies followed by a PE-conjugated anti-human IgG F(ab′)$_2$. The cells were also stained for surface expression of CD45 and CD4, intracellular expression of the viral Gag protein, and with a viability dye. Values indicate the geometric mean fluorescence intensity (gMFI) of Env staining on the surfaces of virus-infected (Gag$^+$ CD4^{low}) cells of the viable CD45$^+$ population. DEN3 is a dengue virus-specific monoclonal antibody included as a control for nonspecific staining.

ADCC activity against HIV-1_{JR-FL}- or $SHIV_{AD8-EO}$-infected cells also neutralized these viruses (Table 1). Furthermore, antibody concentrations for 50% neutralization were generally lower than for 50% ADCC (Table 2). However, in the case of HIV-1_{NL4-3}, a number of instances of ADCC in the absence of detectable neutralization were observed (Table 1), and antibody concentrations for 50% ADCC were sometimes lower than for 50% neutralization (Table 2). Notably, 240D and F240, which recognize epitopes exposed in gp41 that are nonneutralizing, mediated efficient NK cell lysis of HIV-1_{NL4-3}-infected cells (50% ADCC of 0.12 and 0.066 μg/ml, respectively) (Table 2). Overall, these results indicate that most antibodies that are able to bind to Env expressed on the surfaces of virus-infected cells to mediate ADCC are also able to bind to Env trimers on the surfaces of virions to neutralize infectivity. However, neutralization is not always predictive of ADCC, and for lab-adapted viruses such as HIV-1_{NL4-3}, conformations of Env may be exposed on the surfaces of virus-infected cells that render them susceptible to antibody-dependent killing but are not relevant to blocking viral infectivity.

DISCUSSION

Increasing evidence suggests that Fcγ receptor-dependent functions of antibodies are important for the optimal antiviral activity of HIV-1-specific antibodies (8–11, 85). Thus, a better understanding of the relationship between neutralizing and nonneutralizing functions of antibodies is needed to guide the development of immunotherapies and antibody-based vaccines for the treatment and prevention of HIV-1 infection. In the present study, antibodies targeting diverse epitopes of the HIV-1 Env protein were tested for ADCC against cells infected with HIV-1 or SHIV isolates, and their ADCC activity was compared to their ability to bind to Env expressed on the surfaces of virus-infected cells and to neutralize viral infectivity. Consistent with recent findings (86, 87), ADCC activity correlated with Env binding and with neutralization for each of the viruses tested, indicating that these functions are largely overlapping; however, instances of ADCC in the absence of detectable neutralization and neutralization in the absence of detectable ADCC were also observed, revealing differences in Env epitopes exposed on the surfaces of HIV-1-infected cells and virions that confer susceptibility to these antiviral activities.

Sensitivity to ADCC corresponded closely with sensitivity to neutralization for antibody-resistant primary isolates. All of the antibodies that directed ADCC against HIV-1_{JR-FL}- and $SHIV_{AD8-EO}$-infected cells also neutralized these viruses. Thus, most antibodies capable of binding to Env on the cell surface and directing the lysis of virus-infected cells are also able to bind functional Env trimers on virions to block viral infectivity; however, this was not always the case for HIV-1_{NL4-3}. In accordance with the greater exposure of the Env proteins of lab-adapted viruses to antibodies (73–75), HIV-1_{NL4-3}-infected cells were generally more sensitive to ADCC, both in terms of the number of antibodies and the magnitude of responses. Although ADCC corresponded with neutralization for many of the bNAbs, cells infected with HIV-1_{NL4-3} were also susceptible to killing by nonneutralizing antibodies. This was particularly evident for F240 and 240D, which mediated potent ADCC against HIV-1_{NL4-3}-infected cells despite their inability to block viral infection (Fig. 4) (88). ADCC responses were also detected for 98-6 and 126-7, which recognize epitopes exposed in the post-fusion conformation of gp41 (39, 70, 71), and for A32 and C11, which target CD4i epitopes of the gp120 inner domain (66, 67, 89–91). The ADCC activity of nonneutralizing antibodies against HIV-1_{NL4-3}-infected cells suggests that Env epitopes that are not

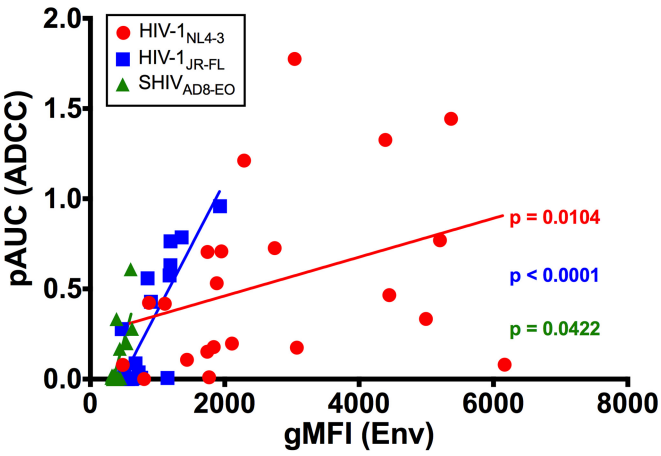

FIG 3 Comparison of ADCC activity and Env binding by HIV-1 Env-specific antibodies. Partial area under the curve values (pAUC) for ADCC activity were calculated from percent RLU measurements at the four highest antibody concentrations tested, as previously described (25, 41). pAUC values for HIV-1_{NL4-3}, HIV-1_{JR-FL}, and $SHIV_{AD8-EO}$ were compared to the geometric mean fluorescence intensities (gMFIs) of Env staining on the surfaces of virus-infected cells by Spearman correlation.

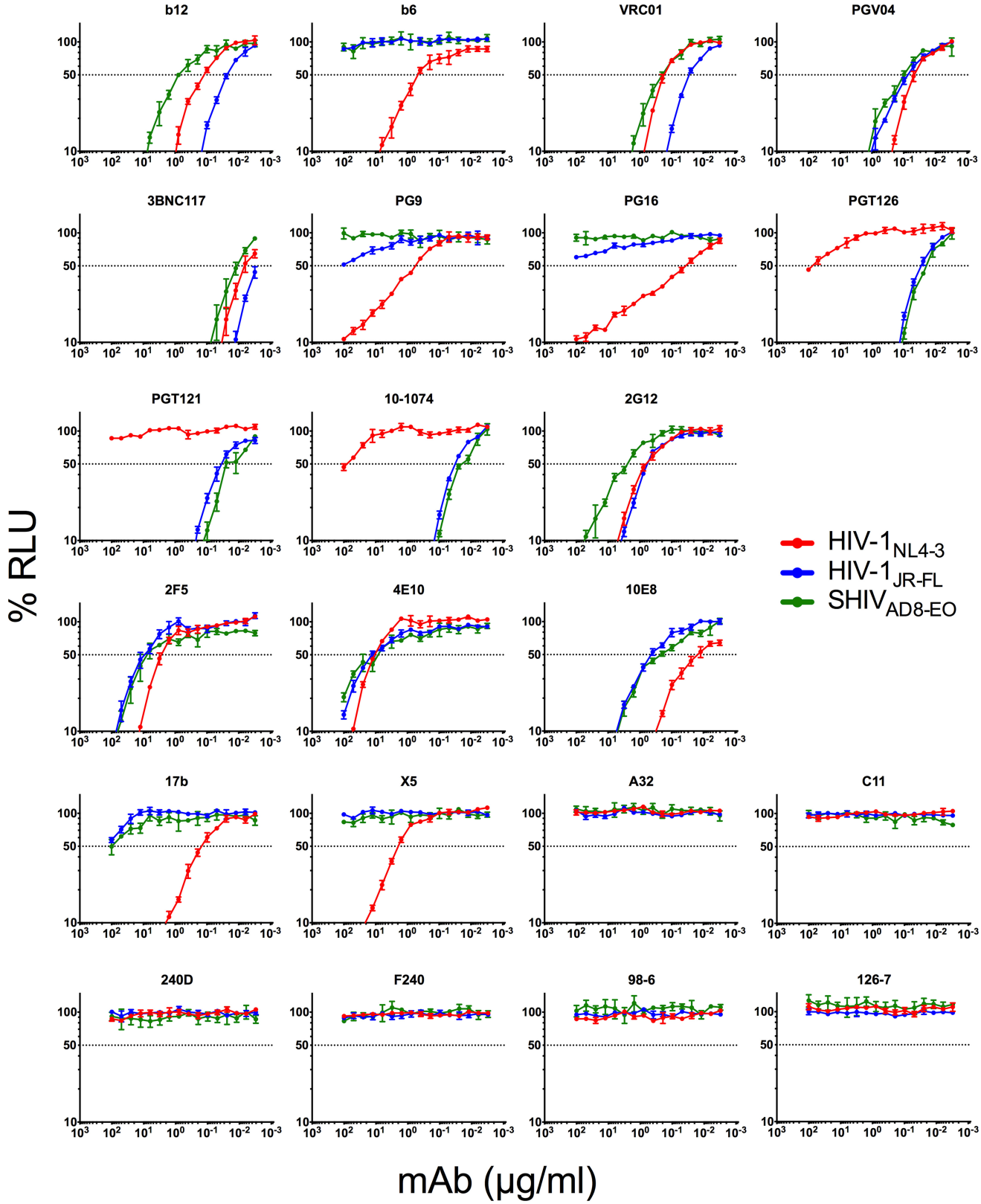

FIG 4 Neutralization of HIV-1_{NL4-3}, HIV-1_{JR-FL}, and $SHIV_{AD8-EO}$ by Env-specific antibodies. HIV-1 Env-specific monoclonal antibodies were tested for the ability to block viral infectivity. HIV-1_{NL4-3}, HIV-1_{JR-FL}, and $SHIV_{AD8-EO}$ were incubated with serial dilutions of each antibody for 1 h before addition to TZM-bl reporter cells. Three days postinfection, neutralization was calculated from the luciferase activity (RLU) in TZM-bl cell lysates for cells inoculated with virus plus antibody relative to cells inoculated with virus in the absence of antibody. The error bars indicate standard deviations of the means for triplicate wells, and the dotted line indicates half-maximal infection or 50% neutralization.

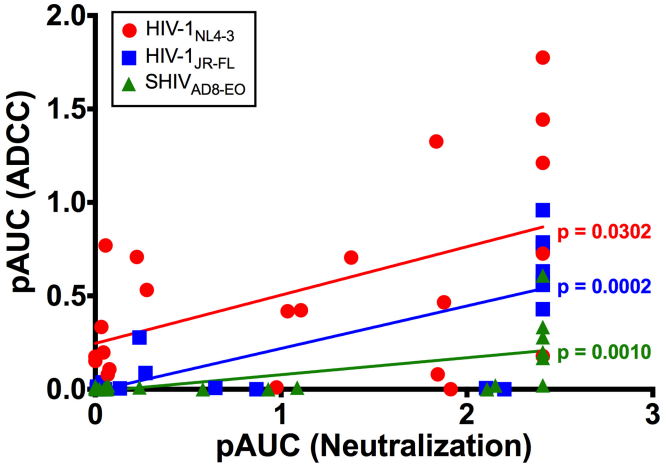

FIG 5 Comparison of ADCC and neutralizing activity of HIV-1 Env-specific antibodies. Partial area under the curve values (pAUC) for ADCC and neutralization were calculated from percent RLU measurements at the four highest concentrations of each antibody, as previously described (25, 41). pAUC values for ADCC and neutralization against HIV-1_{NL4-3}, HIV-1_{JR-FL}, and $SHIV_{AD8-EO}$ were compared by Spearman correlation.

relevant to blocking viral infectivity are exposed on the surfaces of cells infected with lab-adapted viruses that render them susceptible to ADCC.

The striking difference in the susceptibility of HIV-1_{NL4-3}- versus HIV-1_{JR-FL}-infected cells to ADCC illustrates the importance of using primary virus isolates for studying antiviral functions of antibodies. To facilitate virus replication in the face of ongoing immune responses, the HIV-1 envelope glycoprotein has evolved structural features that make it inherently resistant to antibodies (92–95). These features can become attenuated as virus is passaged in T cell lines, accounting for the well-documented increase in the susceptibility of lab-adapted HIV-1 to neutralizing antibodies (73–75, 96, 97). The much greater sensitivity of HIV-1_{NL4-3}-infected cells to opsonization by Env-specific monoclonal antibodies, including antibodies that do not neutralize this virus, suggests that this is also true for ADCC. These observations therefore advocate for the use of primary HIV-1 isolates expressing physiologically relevant conformations of Env on the surfaces of infected cells for studies investigating ADCC or other FcγR-dependent functions of antibodies.

Contrary to earlier reports identifying A32 as a potent mediator of ADCC (98, 99), we found that HIV-1- and SHIV-infected cells are highly resistant to lysis by this antibody. Indeed, ADCC was detected against HIV-1_{NL4-3}-infected cells only at high concentrations of A32, and not at all against HIV-1_{JR-FL}- or $SHIV_{AD8-EO}$-infected cells. Recent evidence suggests that this disparity probably reflects differences in the methods used to measure ADCC. A32 is specific for an epitope on the inner domain of gp120 that is normally occluded in the unliganded Env trimer but can be exposed upon CD4 engagement (53, 94). The accumulation of Env-CD4 complexes on the surfaces of cells infected with viruses deficient for Nef- and/or Vpu-mediated CD4 downregulation was accordingly found to increase exposure of this epitope (100, 101). Shed gp120 released from productively infected cells was also found to sensitize uninfected bystander cells to A32-mediated ADCC (102). These studies help to explain the robust ADCC activity for A32 initially observed using target cells pulsed with soluble gp120 or chronically infected with a Nef-deficient HIV-1 (98). The measurement of NK cell degranulation as a surrogate for the direct lysis of virus-infected cells, which cannot differentiate ADCC responses to virus-infected cells from responses to uninfected cells coated with gp120, may also explain the detection of ADCC activity for A32 against cells infected with HIV-1 isolates that retain CD4 downmodulation (98, 99). In accordance with this interpretation, other recent studies using ADCC assays that directly measure the elimination of virus-infected cells have found that viruses that downmodulate CD4 are resistant to A32-mediated lysis (100, 101, 103). These observations therefore further argue for the use of ADCC assays that directly measure the killing of cells infected with HIV-1 isolates expressing functional accessory proteins and native conformations of Env.

Instances of neutralization in the absence of ADCC include the MPER-specific antibodies 2F5, 4E10, and 10E8, and the glycan-specific antibody 2G12. The lack of ADCC activity for the MPER bNAbs is probably due in part to the lower affinity of these antibodies for Env on virus-infected cells, which is consistent with their specificity for an epitope consisting of gp41 sequences that are transiently exposed during fusion and phospholipids that are preferentially enriched in viral membranes (77–80). Yet these antibodies still bound to HIV-1_{NL4-3}- and HIV-1_{JR-FL}-infected cells, as indicated by levels of Env staining similar to other antibodies with ADCC against these viruses, such as the V2 apex bNAbs PG9 and PG16. The reason for this discrepancy in binding versus ADCC is unclear at this time but potentially reflects the orientation of MPER-specific antibodies bound to gp41, which may hinder their accessibility for engagement by NK cells. The lack of detected ADCC activity for 2G12 was also surprising considering the ability of this antibody to stain Env on the surfaces of HIV-1_{NL4-3}- and HIV-1_{JR-FL}-infected cells. 2G12 is specific for a cluster of high-mannose glycans on the outer domain of gp120 that should not limit its accessibility (56, 104); however, 2G12 has an unusual domain-swapped configuration and propensity for dimerization that may impair FcγR interactions (105, 106). Although monomeric and dimeric forms of 2G12 were shown to mediate ADCC against a cell line expressing HIV-1 HXB2 gp160 (107), our data are consistent with recent reports that have found negligible ADCC activity for 2G12 against HIV-1-infected cells (86, 98, 103).

Overall, our results reveal a general correlation between ADCC and neutralization by HIV-1 Env-specific antibodies, which implies, perhaps not surprisingly, that most antibodies that are able to bind to functional Env trimers on virions to block infectivity are also able to bind to Env expressed on the surfaces of virus-infected cells to direct their elimination by ADCC. This correlation was imperfect, however, as several instances where these antiviral activities did not correspond were observed. These exceptions point to underlying differences in Env epitopes on the surfaces of virions and infected cells that differentiate susceptibility to neutralization versus ADCC. Hence, this study provides new insights into the relationship between neutralization and ADCC that may help to guide the development of antibody-based vaccines and immunotherapies for the prevention and treatment of HIV-1 infection.

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