

Antibody-Dependent Cellular Cytotoxicity against Primary HIV-Infected CD4⁺ T Cells Is Directly Associated with the Magnitude of Surface IgG Binding

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Antibody (Ab)-dependent cellular cytotoxicity (ADCC) is thought to potentially play a role in vaccine-induced protection from HIV-1. The characteristics of such antibodies remain incompletely understood. Furthermore, correlates between ADCC and HIV-1 immune status are not clearly defined. We screened the sera of 20 HIV-1-positive (HIV-1⁺) patients for ADCC. Normal human peripheral blood mononuclear cells were used to derive HIV-infected CD4⁺ T cell targets and autologous, freshly isolated, natural killer (NK) cells in a novel assay that measures granzyme B (GrB) and HIV-1-infected CD4⁺ T cell elimination (ICE) by flow cytometry. We observed that complex sera mediated greater levels of ADCC than anti-HIV-1 envelope glycoprotein (Env)-specific monoclonal antibodies and serum-mediated ADCC correlated with the amount of IgG and IgG1 bound to HIV-1-infected CD4⁺ T cells. No correlation between ADCC and viral load, CD4⁺ T cell count, or neutralization of HIV-1_{SF162} or other primary viral isolates was detected. Sera pooled from clade B HIV-1⁺ individuals exhibited breadth in killing targets infected with HIV-1 from clades A/E, B, and C. Taken together, these data suggest that the total amount of IgG bound to an HIV-1-infected cell is an important determinant of ADCC and that polyvalent antigen-specific Abs are required for a robust ADCC response. In addition, Abs elicited by a vaccine formulated with immunogens from a single clade may generate a protective ADCC response *in vivo* against a variety of HIV-1 species. Increased understanding of the parameters that dictate ADCC against HIV-1-infected cells will inform efforts to stimulate ADCC activity and improve its potency in vaccinees.

Antibodies (Abs) can mediate effector functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular viral inhibition (ADCVI), and phagocytosis through binding of the Fc portion to receptors (FcR) on the surface of cells such as macrophages and natural killer (NK) cells (5, 6). In the case of lentiviral infections, there is now some evidence that virus-specific IgG may mediate these functions *in vitro* and *in vivo* (14). In passive or active immunization studies, these functions are implicated in mediating protection from simian immunodeficiency viruses (SIVs) expressing human immunodeficiency virus type 1 (HIV-1) Env (simian-human immunodeficiency viruses [SHIVs]) by antibodies without neutralizing activity (11, 20, 53). Recently, more direct evidence has come from passive-transfer studies in which the Fc of the b12 monoclonal antibody (MAb) was mutated such that FcR binding was disrupted (16). In passively immunized rhesus macaques, this mutation resulted in a marked decrease in the level of protection observed upon SHIV challenge compared to that provided by the nonmutated antibody. In addition, antibody effector functions mediated through Fc binding are thought to be one possible mechanism mediating protection from HIV-1 infection in humans in the recent Thai RV144 vaccine efficacy trial (37). These observations have led to considerable focus on understanding these effector functions in greater detail.

In the case of ADCC mediated by NK cells, the Fcγ receptor IIIa (FcγRIIIa) on the surface of NK cells binds to the Fc of IgG1 or IgG3 (32). Upon cross-linking of the Fcγ receptor, NK cells release the pore-forming protein perforin, which permits entry of gran-

zymes into the target cell cytoplasm, inducing apoptosis. NK cell-mediated killing of targets has been examined in some prior reports. However, the aim of many of these studies was not to understand the qualities of patient sera that mediate high levels of ADCC. Most prior studies were directed at understanding a specific function of NK cells (4, 6, 22, 28, 42, 43) or antibody (10, 23, 30, 46, 47). To this end, they have examined NK cell-mediated ADCC in the context of MAbs or heterologous cell lines or have measured indirect markers of ADCC such as cytokine expression by NK cells (5, 12, 13). In addition, many prior studies have used protein-pulsed target cells (6, 22). These targets may not closely approximate the situation *in vivo*, in that they have relatively high levels of surface HIV-1 Env, which does not account for variability in conformation or glycosylation, and HIV-1 Env is not presented on the cell surface in a native, receptor-unligated form. Thus, while thought to have a protective role in humans, the characteristics of HIV-1-specific antibodies in patient sera that mediate ADCC remain incompletely understood.

To better understand the characteristics of an effective ADCC-mediating antibody response, we have examined some of the qual-

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ities of HIV-1-infected patient sera that are associated with high levels of ADCC. To accomplish this, we have used primary HIV-1-infected CD4⁺ T cells as targets and unstimulated autologous NK cells as effectors. We observed that high levels of ADCC are not associated with plasma viral load, peripheral blood CD4⁺ T cell count, or neutralizing activity. We also observed no association with antibody affinity or specificity. Rather, high levels of ADCC were most closely associated with high levels of IgG, especially IgG1, binding to primary HIV-1-infected CD4⁺ T cells.

MATERIALS AND METHODS

Study participants. HIV-infected subjects were recruited from the Clinical Research Center, National Institutes of Health (Bethesda, MD), and signed National Institute of Allergy and Infectious Diseases (NIAID) Investigational Review Board-approved informed-consent documents. Sera from 9 long-term nonprogressors (LTNP) and 11 progressors were examined in this study. LTNPs were defined as having set point plasma viral RNA levels of <50 copies/ml, stable CD4⁺ T cell counts, and no opportunistic infection in the absence of highly active antiretroviral therapy (HAART). Progressors had been off HAART for >5 years at the time of sampling, had been diagnosed with HIV >7 years prior to sampling, and had declining CD4⁺ T cell counts or CD4⁺ T cell counts of <400 cells/ μ l. HIV-seronegative donors were recruited from the National Institutes of Health donor apheresis clinic. A single seronegative donor was used for data contained in each figure of this work. These donors were typed for Fc γ RIIIa polymorphisms (1). Results in Fig. 2, 3, and 5 to 7 were from studies performed with cells from a single donor homozygous for a valine at position 158. This polymorphism is associated with greater binding of IgG1 and IgG3 on NK cells and greater ADCC compared to cells from individuals homozygous for phenylalanine at this position (24, 50). Results presented in Fig. 4 were from studies performed with cells from a single donor that was heterozygous at position 158, considered to give intermediate binding to Fc γ RIIIa.

Storage of samples. peripheral blood mononuclear cells (PBMCs) were purified from leukapheresis packs using Ficoll density centrifugation with lymphocyte separation medium (MP Biomedicals, Solon, Ohio). PBMCs were frozen in recovery cell culture freezing medium (Gibco, Carlsbad, CA) using a CryoMed controlled-rate freezer (ThermoForma, Waltham, MA) and stored at -140°C . Pooled (Bpool) serum was assembled by mixing equal parts of sera from 20 broadly neutralizing clade B HIV-infected patients. Normal sera were pooled by mixing equal parts of sera from 10 HIV-negative subjects. All sera were heat inactivated at 56°C for 1 h and stored at -80°C .

Antibodies. A novel human-murine chimeric anti-CD3 antibody was used as a positive control for NK cell-mediated CD4⁺ T cell lysis. This chimeric antibody was constructed by overlapping PCR amplification of cDNA templates encoding the heavy- and light-chain variable regions of the OKT3 antibody from the OKT3 clone (ATCC, Manassas, VA) and the human IgG1 constant region or kappa fragment, respectively, as previously described (15). The coding sequences of the light- and heavy-chain fusions were cloned into a modified retrovirus expression vector, pMSGV-1 (21), that carries the neomycin or puromycin resistance gene, respectively, to construct the expression vectors. The expression vectors were cotransfected into CHO cells, and the clone producing the chimeric antibody was isolated by limiting dilution. Antibody was purified by protein A chromatography. MAbs 2F5 and 2G12 were purchased from Polymun Scientific (Vienna, Austria). b12 and b12-LALA antibodies were produced as previously described (19) and provided by Dennis R. Burton. VRC01, 17b, 447-52D, 2.2C, and 211C antibodies were provided by John Mascola (51). The VRC01 DE variant antibody was provided by Gary Nabel. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 monoclonal antibodies A32 and 48D from James E. Robinson (31, 45, 52). Expression plasmids encoding the 4-116, 4-554, 4-133, 4-221, and 6-129 antibodies isolated from patients 4 and 6 were cloned expressed,

and purified as previously described (40). Briefly, Abs were produced by cotransfecting heavy- and light-chain IgG expression plasmids into 293T cells using Fugene 6 (Roche, Indianapolis, IN). Supernatants were harvested after 3 days and purified on EconoPac columns (Bio-Rad, Hercules, CA) using recombinant protein A Fast Flow Sepharose beads per the manufacturer's instruction (GE Healthcare, Piscataway, NJ). After Ab purification, elution buffer was exchanged 3 times with phosphate-buffered saline (PBS) in Amicon Ultra 30-kDa centrifugal filtration conical (Millipore, Billerica, MA). IgG was purified from B-pool and patient 4 sera using a Montage antibody purification kit with PROSEP-G medium per the manufacturer's instructions (Millipore). Ab yields were often 1 to 5 mg/ml, as determined by enzyme-linked immunosorbent assay.

Virus. Several replication-competent infectious molecular clones (IMCs) were used in this study. Two of them, pCH162.c and pCH067.c, represent the full-length transmitted/founder (T/F) virus sequences inferred from two trial CHAVI 001 subjects infected with clade C HIV-1 (C. Ochsenbauer and J. C. Kappes, unpublished data) and were recently derived by applying methods as described previously (33). Other replication-competent IMCs that we utilized expressed *env* genes of interest derived from clade B, C, or A/E HIV-1 in *cis* in NL4-3-derived proviral backbones (Env IMCs) with or without a reporter gene, using an approach previously described (9, 34): pNL-YU2.ecto, pNL-THRO.ecto, pNL-LucR.T2A-AE.C1081 c03.ecto, pNLENG1i-AE.CM235.ecto, and pNL-96ZM.ecto. SF162 infectious molecular clone was provided by Cecilia Chang-Meyer. For production of murine leukemia virus (MLV) pseudovirus, SV-A-MLV-*env* and pSG3 Δ *env* were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (27, 48, 49). Plasmids were propagated in Stbl2 cells and purified using Endo-Free plasmid maxikits (Qiagen, Valencia, CA) according to the manufacturer's protocols. Each IMC was subsequently transfected into 293T LentiPhos cells using Fugene HD (Roche) according to the manufacturer's protocols. We generated MLV *env* pseudovirus by cotransfecting 293T cells with pSG3 Δ *env* and the SV-A-MLV-*env* plasmid using Fugene 6 (Roche). Supernatant was harvested after 3 days and concentrated 60-fold in Amicon 100-kDa centrifugal filtration conical (Millipore).

TZM-bl cell neutralization assay. Neutralization assays using HIV_{SF162} pseudovirus were performed as previously described (41, 48).

Fluorometric granzyme cytotoxicity assay. Cryopreserved PBMCs were thawed in 10% fetal bovine serum (FBS)–RPMI medium, and CD4⁺ T cells were positively selected using magnetic automated cell sorting (Miltenyi, Germany) according to the manufacturer's instructions. Cells were then stimulated for 3 days in medium containing OKT3, anti-CD28, and interleukin-2 and infected with the virus or pseudovirus of interest using Viro Mag beads (OZ Biosciences, Marseille, France) as previously described (29). This procedure results in 30 to 60% infection of primary targets at 36 h when targets are mixed with effectors. NK cell effectors were negatively selected from PBMCs using magnetic automatic cell sorting (NK cell enrichment kit; StemCell, Vancouver, BC, Canada). Targets were labeled with 0.8 μ l LIVE/DEAD fixable violet stain in 100 μ l PBS, and effectors were labeled with 1 μ l LIVE/DEAD fixable far red stain in 100 μ l PBS (Invitrogen, Eugene, OR). Both targets and effectors were resuspended in a 3 \times dilution of granzyme B (GrB) substrate (GranToxiLux; OncoImmune, Gaithersburg, MD), combined at an effector cell/target cell (E/T) ratio of 10:1, and incubated in the presence of HIV-positive (HIV⁺) serum or Ab in 96-well round-bottom plates at 37°C for 1 h. Cells were harvested into tubes and placed on ice prior to analysis. Samples were analyzed on a FACSaria IIu cytometer (BD Biosciences). From the lymphocyte population, LIVE/DEAD far red stain-positive effectors were excluded and LIVE/DEAD violet stain-positive targets were selected. Intracellular GrB content was assessed in the 2,000 LIVE/DEAD violet stain-positive events collected (Fig. 1). The remaining cells were subsequently fixed with Cytofix/Cytoperm (BD Biosciences) and stained for intracellular viral capsid p24 using KC57-RD1 (Beckman Coulter, Miami, FL) and anti-CD4 phycoerythrin-Cy7 (BD Bioscience) to confirm infected-cell

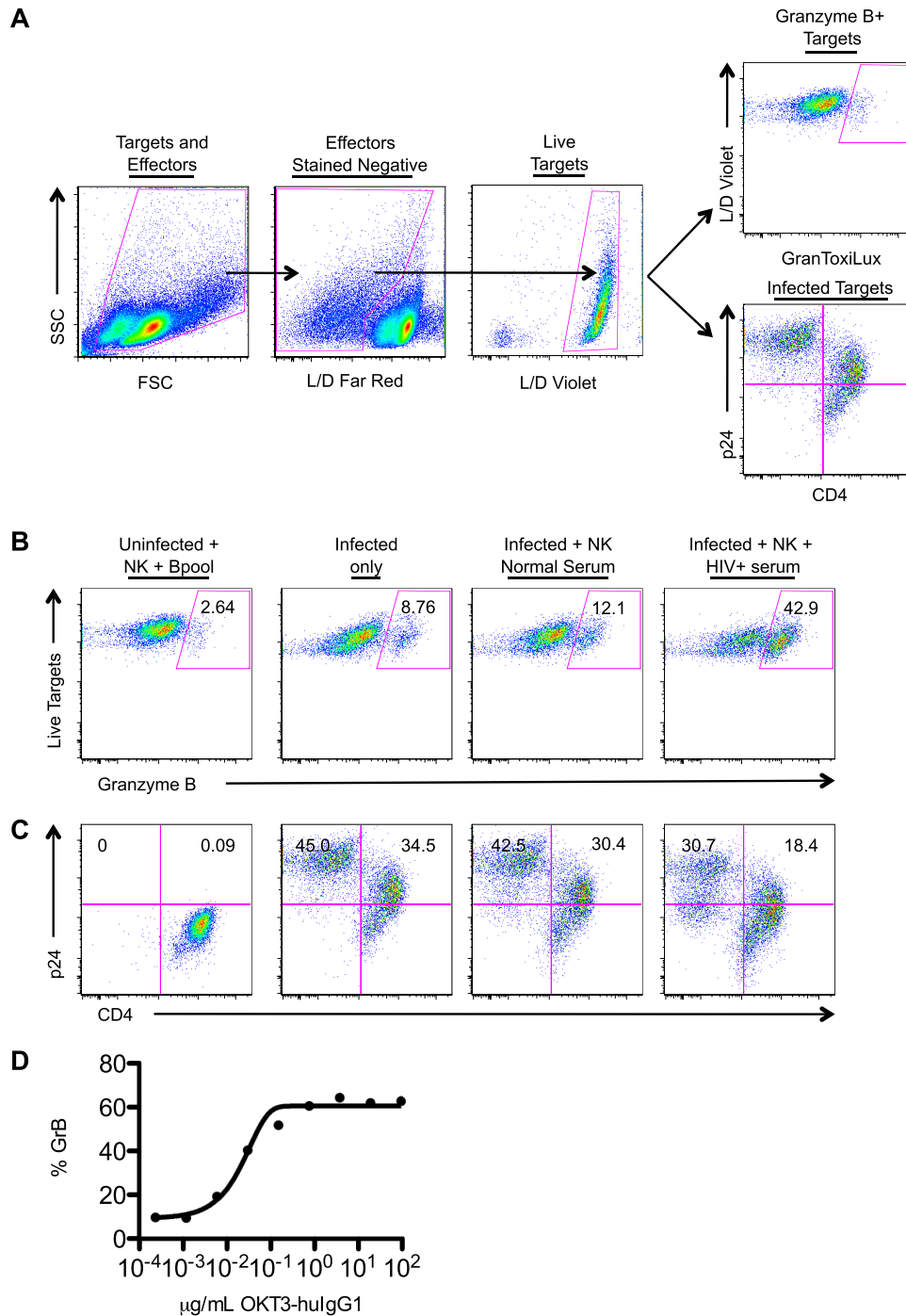


FIG 1 Fluorometric granzyme cytotoxicity assay effectively measures serum-mediated cytotoxicity against CD4⁺ T cells. (A) Gating strategy for granzyme B-positive and infected targets. Live CD4⁺ targets (LIVE/DEAD [L/D] violet stain positive) were selected from the negatively stained NK cell population (LIVE/DEAD far red stain negative). GrB cleaved GranToxiLux substrate, indicating active GrB present within live targets. Live, CD4⁺ targets were also fixed and stained for p24 to determine the frequency of infected targets. SSC, side scatter; FSC, forward scatter. (B) Cellular GrB content. (C) Elimination of HIV-infected cells. Representative data show active GrB in live targets or p24 in CD4⁺ cells. Some infected cells show downregulation of CD4. A reduction in the number of p24-stained cells is represented as ICE and is an indication of cell death. In the example described above, $ICE_{\text{infected-NK cells-serum}} = [(42.5 - 30.7)/42.5] \times 100 = 27.7\%$. (D) A novel human anti-CD3 Ab (OKT3-hulG1) mediates delivery of GrB by NK cells to CD4⁺ T cell-infected targets.

elimination. Data were analyzed using FlowJo software (TreeStar, San Carlos, CA). HIV-1-infected CD4⁺ T cell elimination (infected-cell elimination [ICE]) was calculated as $[(\text{percent p24 expression of infected targets with effectors} - \text{percent p24 expression of infected targets mixed with$

effectors and sera)/percent p24 expression of infected targets with effectors] $\times 100$. Results are reported by subtracting the background of targets-effectors-normal serum for patient serum samples and targets-effectors for MAbs to determine killing attributable solely to the presence of

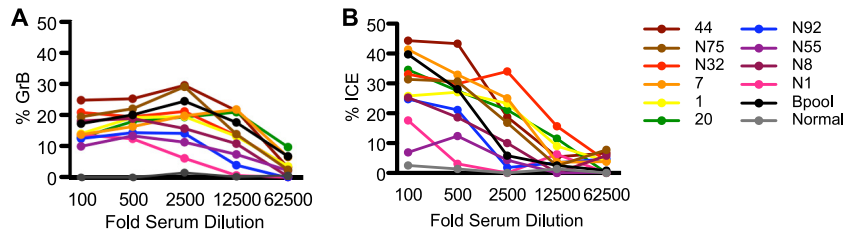


FIG 2 Antibodies mediate ADCC in a dose-dependent manner. GrB delivery (A) and ICE (B) by serum at serial 5-fold dilutions starting at 1:100 for sera from 10 HIV⁺ patients, Bpool serum, and normal serum.

IgG. Experiments were performed in duplicate on 3 separate days with similar results. In preliminary experiments, the coefficient of variation for ICE was 15.2% across 3 days. Each figure in this report shows representative data from 1 day's experiment and for PBMCs from a single donor.

IgG binding isotype. HIV-infected CD4⁺ T cells were washed twice with PBS, incubated with serum or MAb diluted in 10% FBS–RPMI medium at 37°C for 30 min, washed twice with 1% bovine serum albumin (BSA)–PBS, and stained with cell-bound IgG using either anti-IgG peridinin chlorophyll protein (PerCP; BD Biosciences) or anti-IgG1 biotin (BD Biosciences). Streptavidin-allophycocyanin (APC; Jackson ImmunoResearch, Westgrove, PA) was used as a tertiary stain for IgG1 biotin. After staining for IgG or IgG1, samples were washed in 1% BSA–PBS, fixed using Cytofix/Cytoperm, and stained for p24 using KC57-FITC (Beckman Coulter). Samples were analyzed using flow cytometry. The median fluorescent intensity (MFI) in infected targets was quantified.

Statistics. Comparisons of independent groups were made by the Wilcoxon two-sample test. Correlations were determined by the Spearman rank method. The Bonferroni method was used to adjust *P* values for multiple testing.

RESULTS

Fluorometric granzyme cytotoxicity assay measures ADCC activity of sera against primary HIV-1-infected CD4⁺ T cells. To provide a quantitative analysis of ADCC against primary HIV-1_{SF162}-infected cells, we first developed an assay for measuring NK cell-mediated cytotoxicity. Primary CD4⁺ T cell targets were obtained from HIV-1-uninfected controls and infected *in vitro* with HIV_{SF162}. These cells were then mixed with autologous negatively isolated NK cells in the presence or absence of serum from HIV-infected patients (Fig. 1). NK cells from uninfected controls were used to avoid variations in NK cell function associated with viral load or clinical status. ADCC activity was measured in two ways: by delivery of granzyme B (GrB) to target cells and by ICE (29). OKT3 with a human IgG1 Fc was constructed for use as a positive control. Titration of this antibody in this assay showed maximal delivery of GrB activity at 64.2% and 50% activity at 0.023 μg/ml (Fig. 1D). NK cell effectors mixed with uninfected CD4⁺ T cells and pooled sera from patients infected with clade B HIV (B-pool serum) served as our control for HIV specificity and the standard by which we gated for p24-positive cells (Fig. 1C). Infected targets mixed with effectors and normal (HIV-negative) serum served as an additional control for non-HIV-1-specific ADCC. In this example, the uninfected target-serum-effector control had 2.64% GrB delivery. Infected targets had 8.76% GrB and 79.5% infection, as determined by staining for p24. Infected targets, normal serum, and effectors had 12.1% GrB. Adding HIV-1⁺ serum to infected targets-effectors resulted in a 3-fold increase in GrB, 42.9%, and 27.7% ICE.

To further characterize ADCC activity against primary HIV-1-infected cells, GrB and ICE were measured in patient sera over a

range of dilutions. Sera from 10 HIV⁺ patients, B-pool serum, or normal sera were diluted 5-fold from 1:100 to 1:62,500 (Fig. 2). Consistent with some prior work, there was evidence of reduced ADCC activity at the lowest serum dilutions (highest serum concentrations) (10, 11). Peak ICE was observed at a 1:100 dilution for 70% (7/10) of the patient samples. While peak GrB often did not parallel with peak ICE for a given serum sample, GrB and ICE levels followed the same trend. Diluting serum 62,500-fold almost completely ablated ADCC by most sera. ICE and GrB levels were significantly correlated at the 1:500 dilution ($r = 0.75$, $P = 0.04$).

ADCC does not correlate with CD4⁺ T cell count, viral load, or neutralizing antibody (NAb) titers. Some, but not all, prior work has associated the ADCC activity of sera with clinical status, CD4⁺ T cell count, or viral load. It has been suggested that sera from rare individuals who do not progress to AIDS without antiretroviral therapy (elite controllers [ECs] or LTNP) exhibit greater ADCC activity, which correlates with the CD4⁺ T cell count (2, 3, 26), while others have observed no correlation between ADCC and disease progression (7, 25). We sought to determine whether greater HIV-1-specific ADCC was associated with clinical status. We compared the sera of 9 LTNP and 11 progressors (Table 1; Fig. 3A) in our assay using HIV_{SF162}-infected primary CD4⁺ T cells. ICE levels ranged from 19.70% to 40.00% for LTNP and 16.30% to 52.30% for progressors (Fig. 3B). The median GrB levels were 19.80% and 20.05% for LTNP and progressors, respectively ($P > 0.5$). The median ICE levels for LTNP and progressors were 29.80% and 36.90%, respectively ($P > 0.5$). Neither GrB nor ICE was correlated with the CD4⁺ T cell count or viral load ($P > 0.5$ for each correlation) (Fig. 3C and D). If one examines the correlation between viral load and ICE among progressors only, there was also no correlation ($r = 0.33636$, $P = 0.31$). There was also no correlation between ADCC activity and titers of neutralizing antibodies against HIV_{SF162}, the virus used to infect ADCC targets ($P = 0.47$; Fig. 3E), or with the geometric mean of neutralizing titers against 20 tier 2 primary isolates ($P > 0.5$; Fig. 3F) (8).

Serum exhibits breadth in mediating ADCC. We investigated the ability of cells infected with a range of isolates to be killed in this assay. B-pool serum was used to measure ADCC against CD4⁺ T cells infected with isolates from clade B (SF162, THRO, YU2, 96ZM), C (CH162, CH067), and A/E (C1081, CM235) viruses (Fig. 4). Clade B (Bpool) sera were able to kill 35.50% to 73.30% of infected cells from all three clades (ICE for clade B, SF162 = 35.50%, THRO = 60.25%, YU2 = 66.58%, and 96ZM = 41.18%; ICE for clade C, CH162 = 62.8%, and CH067 = 61.65%; ICE for clade A/E, C1081 = 73.3% and CM235 = 49.50%).

Although HIV-1 Env is widely considered the major target for ADCC-mediating antibodies, there are prior reports of Abs spe-

TABLE 1 Patient characteristics

Patient no.	Diagnosis yr	CD4 ⁺ T cell count (no. of cells/ml)	Plasma HIV RNA load (no. of copies/ml)	HLA class I allele:		
				A	B	C
LTNP						
7	1985	401	<50–77	1, 2	57	6
30	1990	229	<50–416	31, 74	51, 57	7, 16
47	1994	407	<50–56	1, 74	57, 81	7, 18
72	1998	1, 287	<50	30, 33	42, 58	2, 17
73	1991	801	<50	2, 3	7, 57	6, 7
74	1988	851	<50	11, 32	35, 50	4, 6
79	1998	501	<50	1, 30	42, 57	7, 17
84	1984	776	<50	3, 26	14, 57	7
93	1986	544	<50	3, 68	14, 35	4, 8
Progressors						
1	1985	545	19, 260	3, 66	7, 58	7
20	1985	1, 069	7, 166	1	52, 57	6, 12
21	1984	497	10, 714	2, 10	8, 27	1, 7
44	1986	754	1, 274	1, 24	27, 37	2, 6
59	1986	633	585	23, 30	7, 57	7, 15
63	1986	1, 096	1, 945	30	57, 81	18
69	2000	717	5, 275	2	44, 57	5, 6
70	1984	584	2, 885	1, 24	8, 27	2, 7
131	1989	578	85, 981	2, 11	35, 57	4, 6
265	1991	478	6, 343	23, 30	15, 41	2, 8
266	1992	235	25, 366	24, 33	35, 42	4, 17

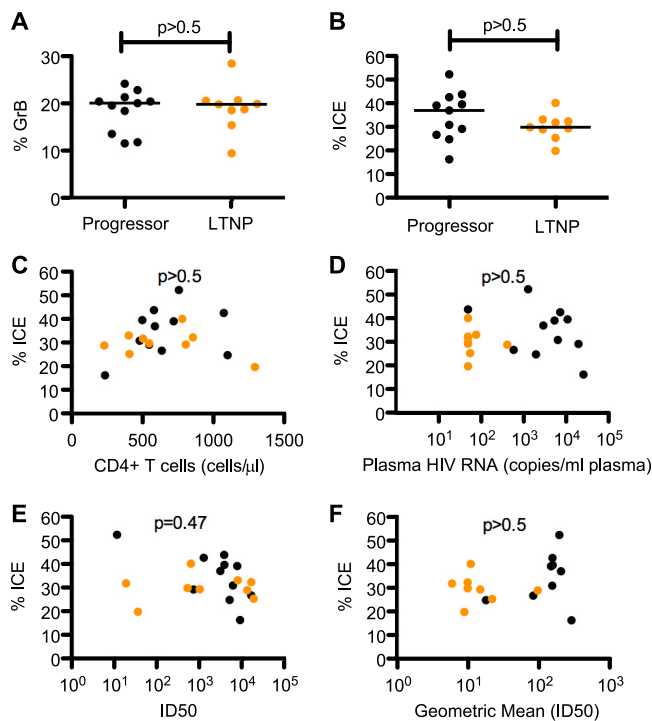


FIG 3 ADCC activity of sera does not correlate with clinical status, CD4⁺ T cell count, viral load, or NAb titers. The ADCC activity of sera from 20 HIV⁺ patients diluted 100-fold was compared by patient status for GrB activity (A) and ICE (B). ICE values for individual patients were also compared to CD4⁺ T cell count (number of cells/μl) (C), viral load (number of copies/ml plasma) (D), NAb titers (50% inhibitory dose [ID₅₀]) against HIV_{SF162} (E), and the geometric mean of NAb titers (50% inhibitory dose) against 20 tier 2 primary isolates (F). Orange, LTNPs; black, progressors.

cific for other HIV-1 gene products mediating ADCC (38). To probe whether recognition of HIV Env by serum Abs was essential for ADCC, we examined killing of targets infected with MLV Env-pseudotyped SG3^{Δenv}. There was a 90.14% reduction in ICE against MLV compared to that obtained with SF162, confirming that most ADCC activity within these sera was Env specific.

Effects of Ab specificity and affinity on ADCC against primary HIV-1-infected targets. In theory, the level of ADCC activity of an HIV-1-specific antibody may be affected by the specificity, affinity, orientation when bound to Env, and/or quantity on the cell surface. Although MAb with a range of specificities have been used in ADCC assays against Env-pulsed cell lines, they have not been widely used against primary HIV-1-infected CD4⁺ T cell targets. Primary cell targets are likely to have less Env antigen on the cell surface and are less easily killed by NK cells than protein-pulsed cell lines (36). We therefore examined the level of ADCC mediated by a panel of well-characterized MABs. Our panel included 13 MABs: 2F5 (anti-gp41); 2G12 (anti-gp120 oligomannose); b12, b12 LALA (mutated to ablate FcR binding), VRC01 (anti-CD4 binding site), and VRC01 DE (mutated to enhance FcR binding); 447-52D (anti-V3); 17B, A32, and 48D (CD4 binding induced); and 2.2C and 211C (anti-gp120 constant region). In addition, we tested a combination of MABs, including 2F5, 2G12, b12, 447-52D, 17B, and VRC01 (Fig. 5A). MABs were serially diluted 10-fold starting at 100 μg/ml to assess their capacity to mediate ADCC. At all dilutions, ADCC mediated by individual or pooled MABs was low compared to that mediated by sera. Neither individual MABs nor a combination of six MABs mediated ADCC to the degree of B-pool serum IgG. Recent reports have cited A32 as being superior to other MABs in directing ADCC (10). In our assay, the ICE mediated by A32 was relatively similar to that mediated by the other MABs. However, it remained well below the maximum ADCC mediated by purified Bpool serum IgG

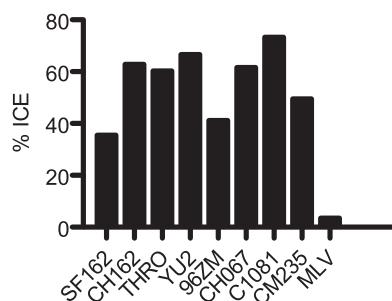


FIG 4 Serum exhibits breadth in mediating ADCC. Bpool serum mediates ADCC against clade B (SF162, THRO, YU2, 96ZM), clade C (CH162, CH067), and clade A/E (C1081, CM235) viruses. MLV is the pSG3^{Δenv} backbone with MLV env.

(43.70%) at 100 $\mu\text{g/ml}$ and was 55.5% less than the maximum ICE of purified Bpool serum at 10 $\mu\text{g/ml}$ (41.8%). The level of ADCC mediated by the VRC01 mutant, known to enhance FcR binding, was also well below that mediated by Bpool serum IgG. Enhanced binding to FcR was confirmed by using the Fc mutant to stain negatively isolated NK cells and detecting surface IgG with a secondary antibody. In these experiments Fc mutants stained a greater fraction of NK cells at all dilutions than the parent MAb or B-pool serum (data not shown). Thus, although Fc mutants have greater binding to NK cells, these mutations did not result in the levels of ADCC observed in individual or pooled sera.

To further explore the specificities and affinity range that may mediate ADCC against primary infected targets, we selected HIV-1-specific MAbs that have been cloned from two patients whose sera exhibited ADCC activity (40). MAbs 4-554 and 4-116 both recognized gp120, but 4-116 binding has been shown to be sensitive to deglycosylation. NABs 4-113 and 4-221 both bind gp120 core, but 4-221 has 50-fold higher affinity (dissociation constants [K_d], $2 \times 10^{-8}\text{M}$ and $2 \times 10^{-10}\text{M}$, respectively). MAb 6-129 is an anti-gp41 nonneutralizing Ab from patient 6. Purified patient 4 IgG and a combination of the four patient 4 MAbs were also tested. Consistent with the results from Fig. 5A, individual MAbs did not mediate high levels of ADCC. The killing mediated by purified IgG from patient 4 far exceeded the killing mediated by individual MAbs or patient 4 MAbs combined (Fig. 5B). Thus, we did not observe a pattern of greater ADCC mediated by individual specificities or Abs of greater affinity. Taken together, these results suggested that individual MAbs or limited combinations of 6 MAbs were unable to achieve the level of ADCC observed using serum-derived IgG and this activity was not related to the level of FcR binding.

ADCC correlates with IgG binding. One possible explanation for the high levels of ADCC mediated by polyspecific sera is binding of larger amounts of total IgG to the surface of HIV-infected cells. Large numbers of specificities may allow binding of multiple Abs per Env spike or binding of Env conformational variants. To explore the contribution of IgG binding to ADCC activity, we titrated patient sera or MAbs on infected CD4⁺ T cells and stained for bound total IgG, IgG1, or IgG3 (Fig. 6A and B). IgG3 staining was very low at all dilutions and was excluded from further analyses (data not shown). To confirm that IgG binding was Env specific, a 1:100 dilution of patient serum was added to uninfected cells, resulting in an MFI of 9.79, whereas infected cells had an MFI of 2,846. We observed a positive correlation between bound IgG

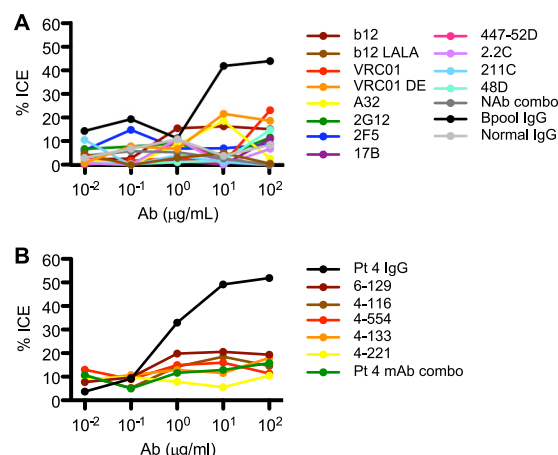


FIG 5 Serum-mediated ADCC is attributable to IgG; single anti-Env MAbs mediate only modest ADCC. (A) Standard neutralizing Abs were titrated in parallel with each other and Bpool serum IgG. (B) Anti-gp120 and gp41 NABs isolated from two patients (patients 4 and 6), a combination of the four patient 4 MAbs, and purified patient 4 IgG were titrated in parallel.

and ICE at a 1:2,500 serum dilution ($r = 0.73$, $P = 0.05$; Fig. 7A). There was also a significant correlation between bound IgG1 and ICE at a 1:100 serum dilution ($r = 0.96$, $P < 0.001$) and a 1:2,500 dilution ($r = 0.81$, $P = 0.01$) (Fig. 7B). In a parallel experiment, the amount of bound IgG was quantified using calibrated beads. We observed that at a serum dilution of 1:100, the median binding was a minimum of 1,123 molecules/cell. In a similar experiment where MAbs were titrated on infected cells and stained for IgG and IgG1, the MAbs bound less to infected cells (Fig. 6C and D). No correlation between MAb binding and ICE was found ($P > 0.5$); however, both values were extremely low in comparison to those for sera. Taken together, reaction of serum with infected cells results in greater IgG binding than reaction of combinations of MAbs, possibly explaining the greater ADCC mediated by serum. Further, within patient sera, ADCC activity was tightly correlated with total IgG or IgG1 binding to infected cells.

DISCUSSION

These results provide some insights regarding the qualities of sera that mediate effective ADCC against primary HIV-1-infected CD4⁺ T cells. In the present study, high levels of ADCC activity were not associated with plasma viral load or CD4⁺ T cell count. In contrast to some prior reports, we were unable to detect greater ADCC activity in sera from LTNP/ECs under our experimental conditions (2, 3, 22, 26). Further, we found no association with neutralization of the virus used in the ADCC assay or with breadth of neutralization. Using single MAbs we were also unable to detect a clear association with affinity or specificity. MAbs mediated very modest or low ADCC activity compared to sera from HIV-1-infected patients. The low ADCC activity of some sera and of most MAbs was associated with lower binding of HIV-1-specific antibodies to the surface of infected cells. Thus, under our experimental conditions, total binding of Env-specific IgG, especially IgG1, tightly correlated with ADCC activity. These results suggest that the total amount of IgG bound to an HIV-1-infected cell is an important determinant of the level of ADCC activity.

The finding that ADCC activity is simply associated with total IgG binding may be surprising at first inspection. However, these

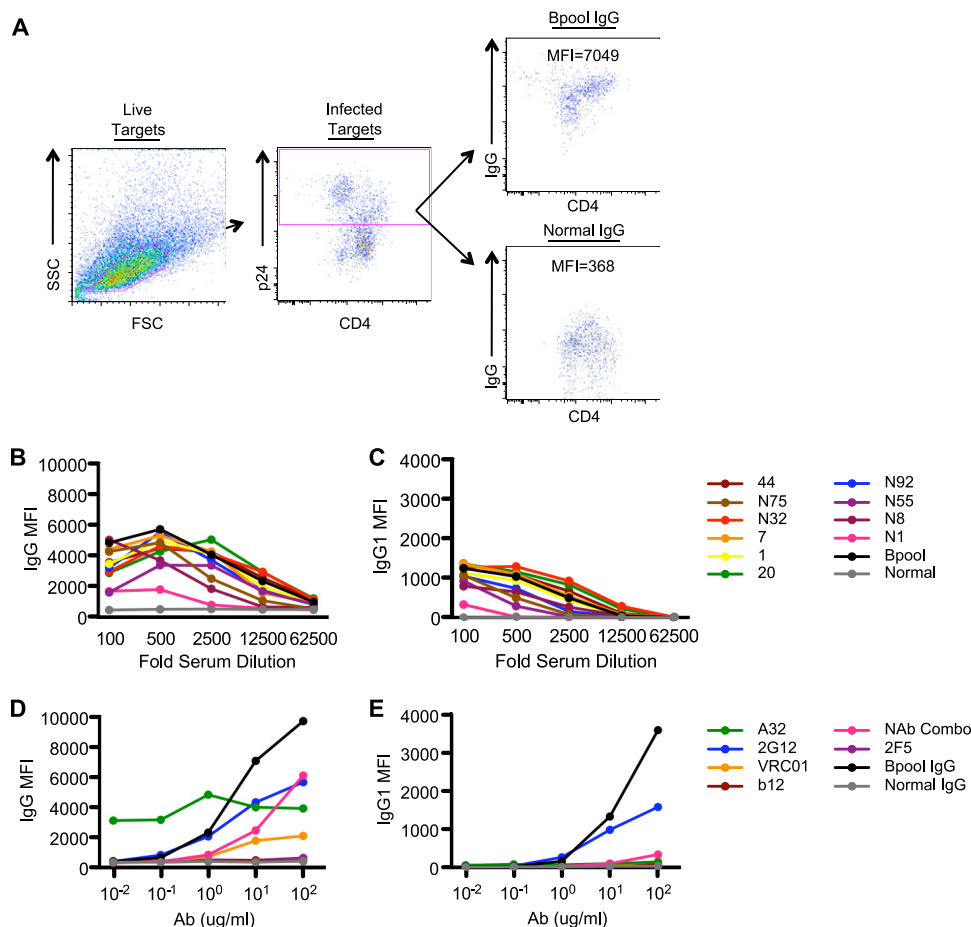


FIG 6 ADCC correlates with surface-bound IgG. (A) Gating strategy to determine surface IgG binding. MFI of anti-IgG (B) and anti-IgG1 (C) bound to serum-coated infected cells; MFI of anti-IgG (D) and anti-IgG1 (E) bound to MAbs whose titers on infected cells were determined (D).

results are potentially consistent with those of a recent study by Sun et al. that showed a correlation between NK cell-mediated ADCC and titers of SIV_{mac251} gp140 binding antibody in the first 14 weeks of rhesus macaque SIV infection (44). Although it is unclear exactly how many antibody molecules must bind to an infected cell to trigger degranulation of an NK cell, most estimates predicted that this number was relatively small. If this were the case, one might expect that binding by only a few IgG molecules of an MAb or from patient sera would mediate high levels of ADCC. If surface Env trimers were uniform, one might also expect that high levels of ADCC would be achieved by combining MAbs with different specificities. However, this was not observed even in combinations of 6 broadly neutralizing MAbs with specificities

that are widely spaced on the Env trimer. Given the size of IgG molecules relative to the Env trimer, it seems unlikely that additional specificities would result in greater IgG binding. Although modification of the Fc to enhance FcγRIII binding increased ADCC activity, it did not attain the level of complex sera. This result suggests that an important limiting factor is not FcR binding but, rather, is at the level of antigen-specific binding on the target cell. We consistently observed the highest ADCC activity using sera from infected patients, and this was associated with greater HIV-specific IgG1 binding than MAb binding. Consistent with this, among patient sera, greater ADCC activity was closely associated with higher IgG1 binding to HIV-infected cells. One likely explanation for the higher ADCC activity with sera than monoclonal antibodies is that Env is not homogeneous and sera containing multiple specificities are better able to recognize various surface conformations, including nonfunctional spikes. These Env forms would include Env with different levels of glycosylation, triggered Env spikes in which the conformation is altered after receptor binding, shed gp120 bound to CD4 on target cell surfaces, and residual gp41 stumps remaining after shedding of gp120.

One might expect that if Env-specific binding is required for ADCC, neutralization might correlate with ADCC, as neutralization is primarily determined by occupancy of the functional Env trimer by antibody (35, 39, 54). However, we did not observe a

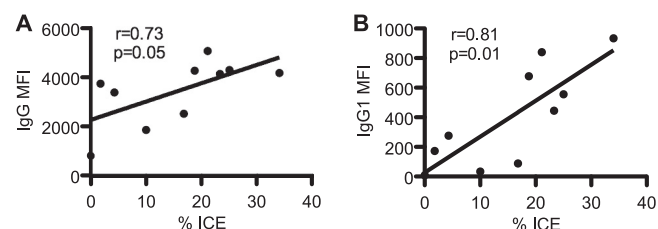


FIG 7 Correlation of ADCC with binding of serum IgG (A) or IgG1 (B) to infected targets. Cells were incubated with patient sera diluted 1:2,500.

correlation between ADCC and neutralization. One possible explanation is that major variations in ADCC are associated with various levels of binding, nonneutralizing antibodies (11). Another possible reason for our inability to detect a correlation between ADCC and neutralization may reflect the fact that some ADCC, measured *in vitro*, is potentially mediated by Abs recognizing non-Env epitopes (38), although our results using an *env*-deletion mutant show that ADCC responses are predominantly directed against Env.

In contrast to some prior work, we also did not observe an association between viral load and the ADCC activity of sera (2, 3, 22, 26). One prior study observed considerably greater ADCC activity of sera from LTNP/ECs than progressors (26). That prior study used very different experimental conditions, including protein-coated cell line targets and chromium release. Likewise, in other studies, no correlation between ADCC and disease progression was observed (7, 25). At present, the precise reason for the disparate results remains unclear.

The results of the present study also have some implications for ADCC mediated in passive-transfer studies. Passive-transfer studies examining the relative contributions of ADCC, ADCVI, or phagocytosis in protection of rhesus macaques from SIV infection have used monoclonal antibodies alone or in combination (17, 18). However, under our *in vitro* conditions, the level of ADCC mediated by MAbs against primary HIV-1-infected cells was only a fraction of that mediated by most sera from chronically infected patients. Although this result is in need of confirmation *in vivo*, it suggests that compared to passive transfer of IgG from complex sera, use of MAbs may underestimate the contribution of ADCC to sterilizing immunity.

A further understanding of the parameters that dictate ADCC-mediated killing of infected cells may have implications for efforts to stimulate ADCC activity in vaccinees. Specifically, it will be important to further define the relative importance of individual specificities versus the ability to recognize structural variants on the cell surface. Isolation of additional antibodies capable of recognizing a broad array of HIV-1 Envs may uncover specificities that are particularly good targets for ADCC. Alternatively, the high levels of ADCC observed using sera may not be mediated by antibodies specific for conserved regions and may be mediated only by the ability to recognize structural variants. If this is the case, then immunization with multiple Env variants may be required to achieve these levels. It is encouraging that some sera from RV144 mediated ADCC in the range of those from chronically infected patients in assays using HIV-1-infected cell lines or primary CD4⁺ T cells (A. Smalls-Mantey and G. Ferrari, unpublished observations). A further understanding of the basis of ADCC-mediated protection through passive-transfer studies in either macaques or vaccinated humans should yield important information for improving ADCC activity and possibly enhancing vaccine potency.

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