CHAPTER 4

Potent neutralization of HIV is not a predictor of the ability to trigger antibody-dependent cellular cytotoxicity

This work was completed in collaboration with my colleague, Alexandre Webster, with assistance from Priyanthi Gnanapragasm and Jost Vielmetter. Using a panel of anti-HIV antibodies, it was observed that their respective abilities to trigger antibody dependent cellular cytotoxicity varied from highly effective to completely ineffective despite the fact that each of the antibodies exhibited potent neutralization of a virus of the same strain as used for the cytotoxicity assay.

Introduction

Certain classes of immunoglobulins can prevent or contribute to the elimination of a viral infection by other mechanisms in addition to neutralization (*i.e.,* binding directly to virus particles to prevent entry into target cells). These additional mechanisms include antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, and activation of the "classical" pathway of complement. With respect to the humoral immune response, the cell-mediated responses of ADCC and/or phagocytosis can be triggered by the interaction of antibody bound to antigen on an infected cell and Fc receptors expressed on the surfaces of various types of innate immune effector cells including natural killer (NK) cells, macrophages, neutrophils, and subsets of γδ T cells (1, 2). Complement activation is triggered by the binding of soluble factors in the plasma to antibody-antigen complexes, beginning with the fixation of C1q, which activates a cascade of reactions that culminate in the formation of pores resulting in lysis of infected cells as well as direct viral inactivation (1, 3). Each of these mechanisms has been shown to play significant roles in controlling and/or eliminating various pathogens (4), but relative to the CTL response and neutralization, the contributions of these responses to the control of HIV replication or their potential value to the prevention of infection remains largely uncharacterized.

Mechanism of ADCC

Of the four subclasses of IgG, only subclasses 1 and 3 are able to trigger ADCC activity in humans (1), which occurs via cross-linking CD16 (FcγRIII), a low micromolar affinity activating Fc receptor (5). CD16, which is a 50-80 kDa highly glycosylated

protein, is expressed as two isoforms: a transmembrane anchored form with a cytoplasmic domain (CD16a) and a glycophosphatidylinositol (GPI) linked form (CD16b) (6). Whereas the precise role of CD16b is not yet fully understood, the function of CD16a on NK cells and macrophages in mediating ADCC is well established (2, 6, 7). Cross-linking of CD16a by antibodies serves to increase the concentrations of the intracellular immunoreceptor tyrosine-based activations motifs (ITAMs) of heterodimers and/or homodimers that belong to the CD16a-associated signaling chains, FcεRIγ and CD3ζ (8), and the resulting protein tyrosine kinase cascade leads to granule exocytosis.

Apoptosis, or programmed cell death, is induced during ADCC following a similar series of steps that describe the mechanism of CTL attack: cytotoxic granules are released from the cytosol of effector cells containing perforin, granzymes, and granulysin (1). Perforin acts in a similar manner to the C9 component of the complement cascade in that it creates pores in the cytoplasmic membrane of the target cell, and granzymes, which then gain entry to the cytosol of the target cell, activate the caspase cascade, culminating in apoptosis (1). However, whereas individual CTLs are specific for individual peptide-MHC class I complexes presented on the surfaces of cells, effector cells that mediate ADCC recognize the Fc domains of bound IgG1 or IgG3 and are therefore competent to attack any infected cell that is expressing a recognized antigen on its surface.

Evidence for a contribution of ADCC to HIV control

Given the lack of evidence that neutralizing antibodies significantly contribute to the control of viral replication during the natural course of infection, researchers are

turning their attention to the role that the humoral immune response might play in recruiting Fc receptor-dependent mechanisms of eliminating infected cells (3, 9-12). This interest has been at least partly fueled by the observations that some antibodies that cannot neutralize virus can still recruit these mechanisms and that these antibodies may appear well before the development of neutralizing antibodies (3). In addition, it has been reported that the ADCC activity titer of sera collected from SIV challenged NHPs vaccinated against SIV is inversely correlated with viral titer and directly correlated with $CD4^+$ T-cell concentrations (10).

Further underscoring the need to carefully examine the contributions of these mechanisms to the control of HIV, ADCC was recently shown to be critical to the ability of IgG1 b12 to protect NHPs from viral challenge (12). When this study was first carried out with the observation that a dosage of 25 mg/kg was necessary to completely protect animals challenged with $SHIV_{SF162P3}$ (13), the conclusion was understood at the time to be that b12 had fully neutralized the virus at this dosage. However, when researchers repeated the study six years later but instead made two different mutants of b12, one that could not fix complement and another than could not bind CD16, the dosage of 25 mg/kg was no longer sufficient for the non-CD16-binding mutant to protect the animals (12). Thus, even when more than 5% of the total IgG in the blood is composed of a monoclonal antibody to which the challenge virus is unusually sensitive, it can still not be enough to fully protect from viral challenge. Further elucidation of the potential contribution of antibodies to viral control by mechanisms other than neutralization may eventually alter the currently accepted view that the humoral immune response is negligible relative to the CTL response in controlling the natural course of infection.

The results presented here are directed towards asking (*i*) if the ability to potently neutralize HIV necessarily implies the ability to efficiently recruit ADCC and (*ii*) if antibodies directed against one region of the envelope spike versus another might result in differing abilities to trigger this mechanism. If ADCC is a critical component to protection, then the possibility that various epitope classes might differ with respect to the ability of antibodies that are directed against them to recruit ADCC should be examined. For example, anti-CD4 binding site (anti-CD4bs) antibodies might be better able to recruit ADCC than antibodies that bind the MPER as a result of greater accessibility to the Fc domain by CD16a. Towards this end, an *in vitro* ADCC assay was developed in which ^{35}S-labeled target cells stably expressing envelope spikes are incubated with antibody and peripheral blood mononuclear cells (PBMCs) and then examined for the release of ^{35}S into the media. The abilities of a panel of bNAbs to neutralize HIV and trigger ADCC were compared, and the implications of these results on vaccine design are discussed.

Results

Selection of a gp160-positive cell line. Three different cell lines that were putatively positive for stable expression of gp160 were obtained from the NIH AIDS Research and Reference Reagent Program (CHO-gp160 (14), HeLa 69T1RevEnv (15), and Jurkat 7/3-STOP (16)), and a fourth was kindly provided by Dr. Pin Wang (USC). Flow cytometry analysis of each of the cell lines stained with FITC-conjugated polyclonal goat anti-gp120 antibody revealed only one cell line to be uniformly expressing gp160: CHO-gp160 (Fig. 1). This cell line was then selected as the basis for the development of the

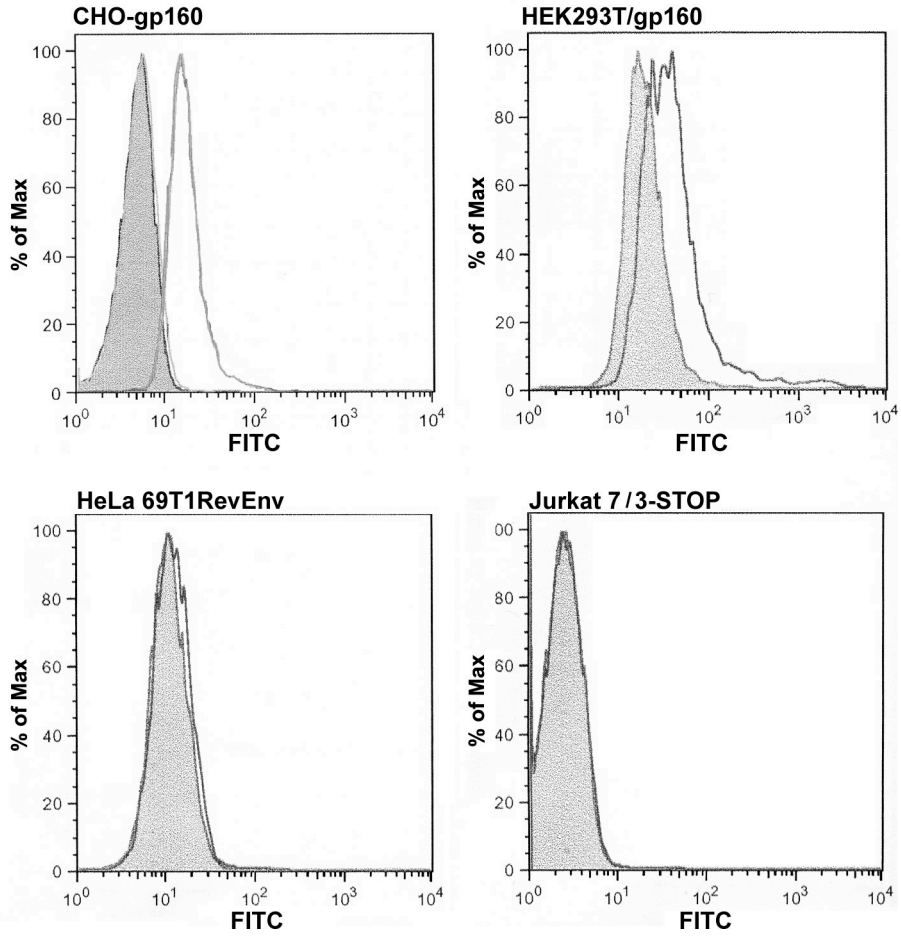

Figure 1. Four cell lines examined for stable surface expression of gp160. Cells were stained with 1 μg/mL FITC-conjugated polyclonal goat anti-gp120 antibody and examined by flow cytometry analysis (grey, unstained; clear, stained).

ADCC assay. A panel of broadly neutralizing IgG1 antibodies including 2G12 monomer, 2G12 dimer, b12, 447-52D, 4E10, and 2F5 were then evaluated for their abilities to neutralize strain HxBc2 (also called IIIB), a T-cell line adapted clade B strain of HIV that is particularly sensitive to each of these antibodies. The antibodies were then evaluated for their abilities to trigger ADCC against CHO-gp160.

Conditions for the ADCC assay included a 30-minute pre-incubation period of antibody with target cells on ice followed by the addition of human PBMCs and incubation for 4 hours at 37 °C at an effector-to-target cell ratio of 50:1. The addition of a monoclonal anti-CD20 isotype control antibody, Rituxan, did not result in non-specific lysis (Fig. 2). In all cases where ADCC activity was observed, the percentage of specific lysis either began to level off or decrease at concentrations above ~10 μg/mL (Fig. 2), probably reflecting increased competition for CD16a between bound and unbound IgG1.

Evaluation of anti-gp120 monoclonal antibodies. Monomeric 2G12 was observed to be effective at recruiting ADCC *in vitro* with a half maximal effective dose (ED_{50}) of 1.1 μg/mL, which was comparable to its 50% inhibitory concentration (IC_{50}) for the *in vitro* neutralization assay of 0.36 μg/mL (Table 1). In agreement with previous results (Appendix F), dimeric IgG1 2G12 was observed to be more potent than the monomer, with ED_{50} and IC_{50} values of 0.32 μg/mL and 0.032 μg/mL, respectively. The performance of these anti-carbohydrate antibodies are unlikely to have arisen from non-specific reactivity as no significant specific lysis was observed when tested against the untransfected parental cell line, CHO K1 (Fig. 3).

The anti-CD4bs antibody b12 exhibited potent ED_{50} and IC_{50} values of 0.058 μg/mL and 0.033 μg/mL, respectively (although the low Hill coefficient of the curve precluded an accurate determination of the ED_{50} value) (Table 1 and Fig. 2). However, the maximal lysis observed for b12 was only 23%, significantly lower than that observed for monomeric 2G12 or dimeric 2G12 (75% and 63%, respectively). It is unlikely that this weak maximal lysis was due to antibody-induced shedding of gp120 as almost no detectable shedding was observed in the presence of b12 and other CD4bs antibodies from the surface of HIV-infected cells in a previous study whereas soluble CD4 (sCD4) was shown to induce the release of over 5 μg of gp120 in 2 hours under identical conditions (17).

The anti-V3-loop antibody, 447-52D, exhibited ED_{50} and IC_{50} values of 0.20 μg/mL and 0.083 μg/mL, respectively. As compared to b12, a similar result was observed in the ADCC assay for 447-52D, with a low maximal lysis value (22%). However, unlike the case for anti-CD4bs antibodies, anti-V3 loop antibodies have been shown to efficiently induce gp120 shedding at rates that are nearly comparable to sCD4 for T-cell line adapted strains of HIV (17), rationalizing its poor performance in the ADCC assay.

The anti-MPER antibodies 4E10 and 2F5 do not exhibit ADCC activity. By contrast to the anti-gp120 antibodies, 4E10 and 2F5 were unable to elicit detectable ADCC activity at any of the concentrations tested even though both were extremely potent in virus neutralization (Fig. 2), yielding IC_{50} values of 0.064 μg/mL and 0.026 μg/mL, respectively.

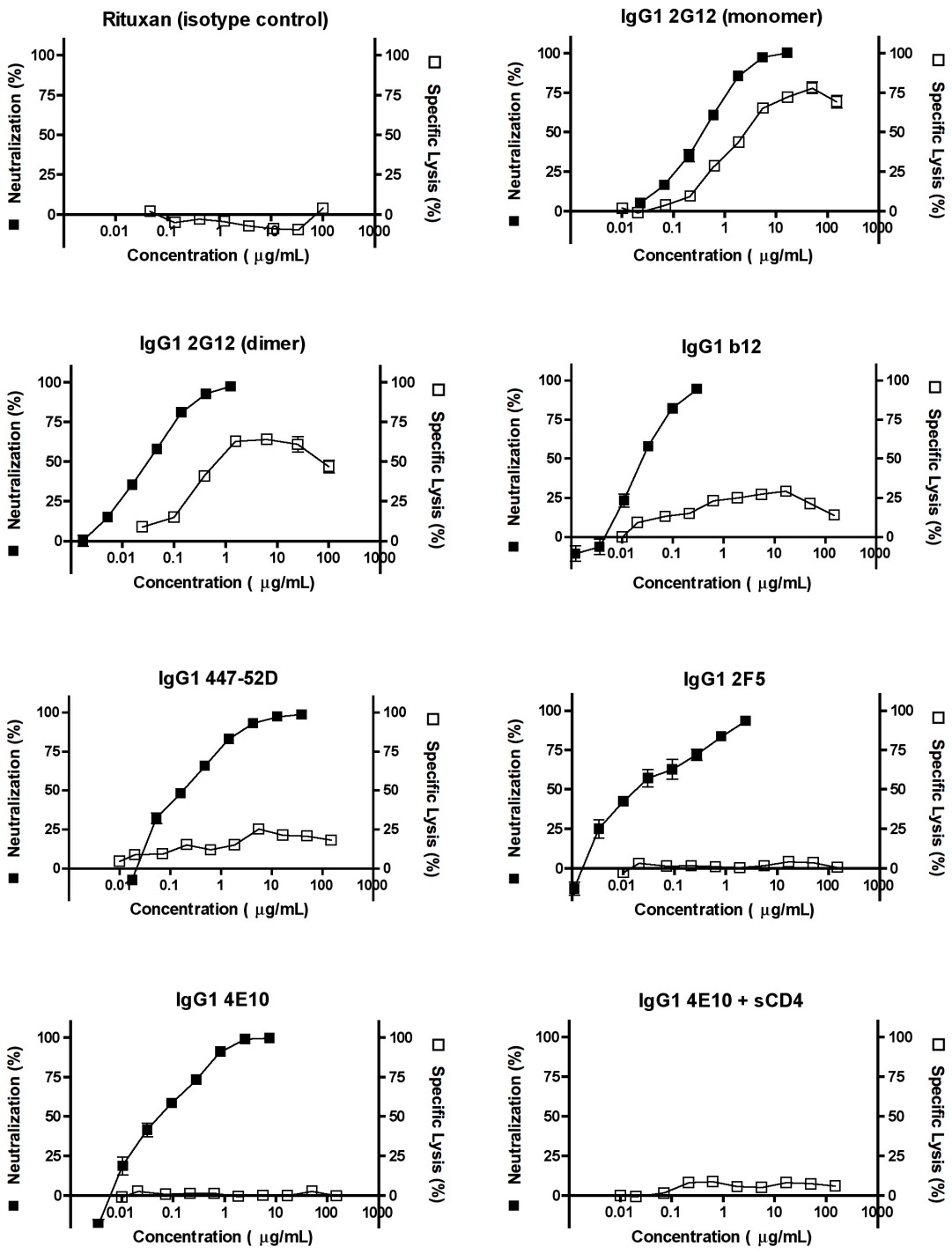

Figure 2. Summary of curves for the *in vitro* neutralization data (■) and *in vitro* ADCC data (□).

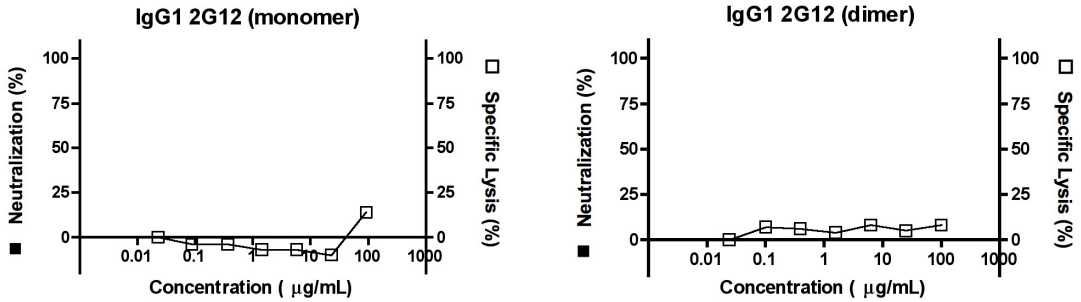

Figure 3. Summary of curves from the *in vitro* ADCC data for control tests of IgG1 2G12 monomer and dimer versus untransfected CHO cells (□).

Table 1. Summary of calculated 50% effective doses (ED_{50}) for specific lysis in the *in vitro* ADCC assay and 50% inhibitory concentrations (IC_{50}) for the *in vitro* neutralization assay (n.d., not done).

	ADCC		Neutralization
Antibody	**EC_{50} (μg/mL)**	**Max lysis**	**IC_{50} (μg/mL)**
Rituxan (IgG1 isotype control)	No activity		n.d.
IgG1 2G12 (monomer)	1.1 ± 0.2	75%	0.36 ± 0.11
IgG1 2G12 (dimer)	0.32 ± 0.05	63%	0.032 ± 0.007
IgG1 b12	0.058 ± 0.125 *	23%	0.033 ± 0.014
IgG1 447-52D	0.083 ± 2.6 *	22%	0.20 ± 0.04
IgG1 4E10	No activity		0.064 ± 0.017
IgG1 4E10 + sCD4	No activity		n.d.
IgG1 2F5	No activity		0.026 ± 0.010

* The weak ADCC activity demonstrated in these curves prevented an accurate determination of their ED_{50} values.

Some data suggest that the addition of sCD4 may potentiate access to the 4E10 epitope (18), but when sCD4 was added at an equimolar concentration to 4E10, no significant increase in activity was observed (Fig. 2), indicating that anti-MPER antibodies in general may be unable to trigger ADCC.

Discussion

In this investigation, the performance of a panel of bNAbs was compared using an *in vitro* neutralization assay and a newly developed *in vitro* ADCC assay. All of the antibodies were potent neutralizers of the strain of virus used (HXBc2), with each one able to neutralize ~100% of the virus at concentrations at or below ~10 μg/mL. However, the antibodies exhibited wide variation in their abilities to recruit ADCC activity.

Monomeric and dimeric IgG1 2G12 were particularly effective at recruiting ADCC, yielding typical sigmoidal dose-response curves and maximal specific lysis values of 75% and 63%, respectively. Importantly, these data confirmed that the Fc domains of dimeric IgG1 2G12 can bind CD16 and efficiently recruit ADCC with enhanced potency over monomeric IgG1 2G12. By contrast, IgG1 b12 and IgG1 447-52D exhibited only modest abilities to recruit ADCC. However, perhaps most striking was the absence of any detectable ADCC activity upon the addition of IgG1 4E10 or 2F5, which target adjacent epitopes of the MPER at the base of the stalk of the trimeric envelope spike. Even with the addition of sCD4, which has been shown to potentiate neutralization by 4E10 (19), presumably by promoting access to its epitope or expanding the time period during which it is available for binding, no detectable ADCC activity was observed.

One explanation that may help to rationalize the observed differences in ADCC activity is the requirement for access by CD16a, a single pass transmembrane protein composed of two immunoglobulin domains that adopt a U-shaped conformation (20, 21). An atomic resolution X-ray crystal structure of the extracellular portion of CD16a in complex with human Fc shows that the CD16a binding site is located on the upper portion of the CH_2 domain (20), which likely places constraints on the rotational freedom of the Fabs (Fig. 4). But perhaps more importantly, the C-terminal sequence that links the second domain of CD16a to the transmembrane sequence is 16 residues long (2). Thus, in the unlikely event of adopting a fully extended conformation, the base of the second domain of CD16a is limited to a separation distance from the effector cell's membrane of ~6.5 nm (Fig. 5).

Given that the trimeric envelope spike extends ~12 nm from the membrane and the lobes created by the gp120 moieties extend several nanometers from the stalk (22), binding to different epitopes may impose demands on CD16a accessibility that cannot be met in some cases (Fig. 5). For example, whereas 2G12 may be binding one or more glycans on the upper portion of spike (23), and b12 binds roughly parallel to the spike (24), anti-MPER antibodies such as 4E10 and 2F5 bind near the base (25, 26). Models of these binding modes indicate that even a fully extended CD16a receptor might have difficulty extending far enough to reach an Fc domain when the antibody is targeting the stalk portion of the envelope spike (Fig. 5), particularly in the presence membrane proteins that extend out from the surface of a typical cell (Fig. 5*D*). Consistent with this hypothesis, in the original paper where 4E10 and 2F5 were first characterized (along with many other antibodies), only 4E10 and 2F5 were reported to derive from IgG3 subtypes (27).

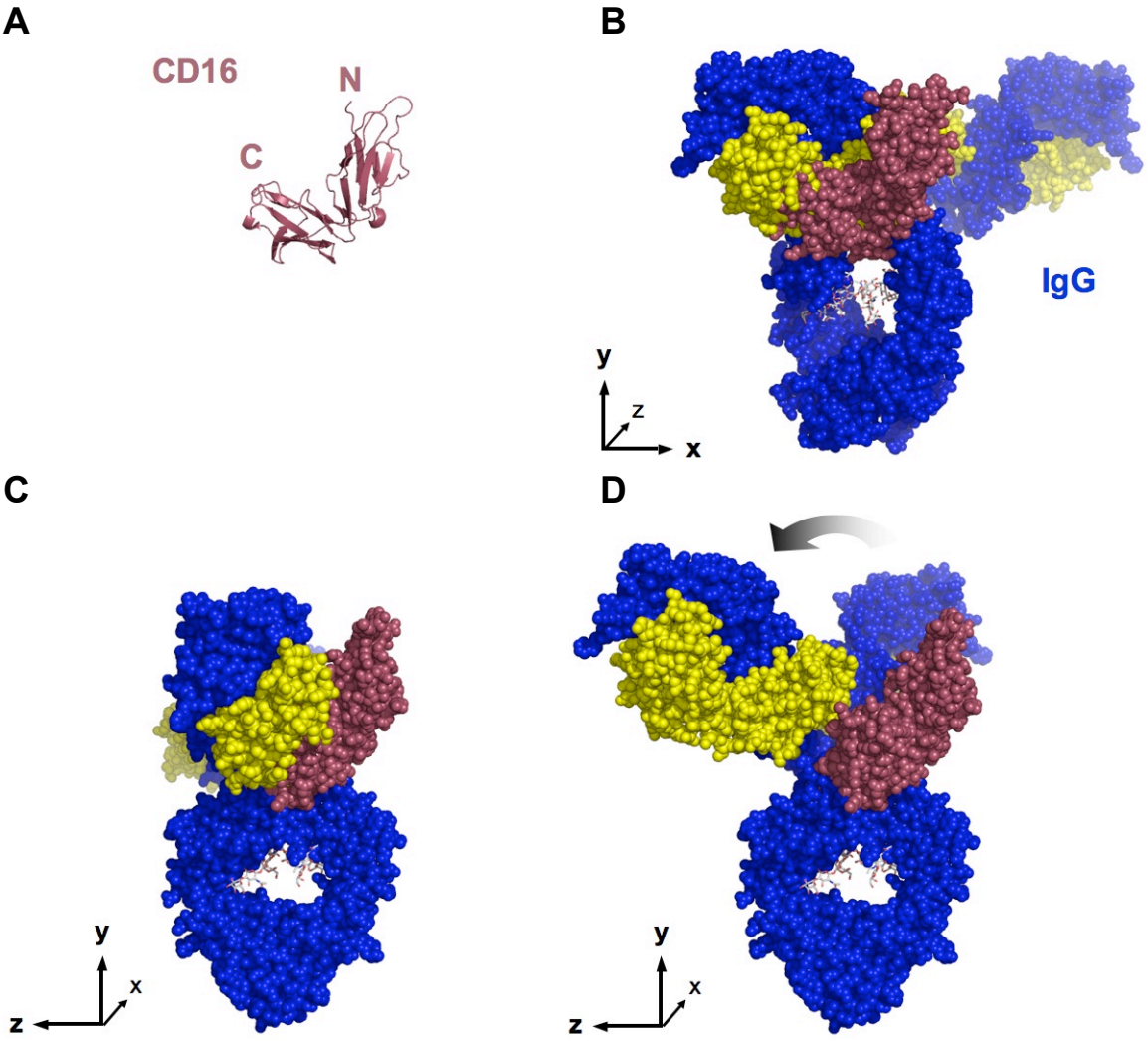

Figure 4. Model structure of IgG-CD16 complex. (*A*) Ribbon diagram for the X-ray crystal structure of CD16 extracellular domain in complex with human Fc (not shown) (PDB ID code 1E4K (20)). (*B*) X-ray crystal structure of IgG1 b12 (PDB ID code 1HZH (28)) docked to CD16. The Fc domains from the two PDB files were used for alignment. (*C*) Same as figure *B* but rotated 90°, showing simultaneous occupancy of space between the Fabs and CD16 when the structures are unaltered. (*D*) Same as figure *C* but the Fabs have been rotated to eliminate clashes between CD16 and the antibody, demonstrating that CD16 binding may impose constraints on Fab flexibility.

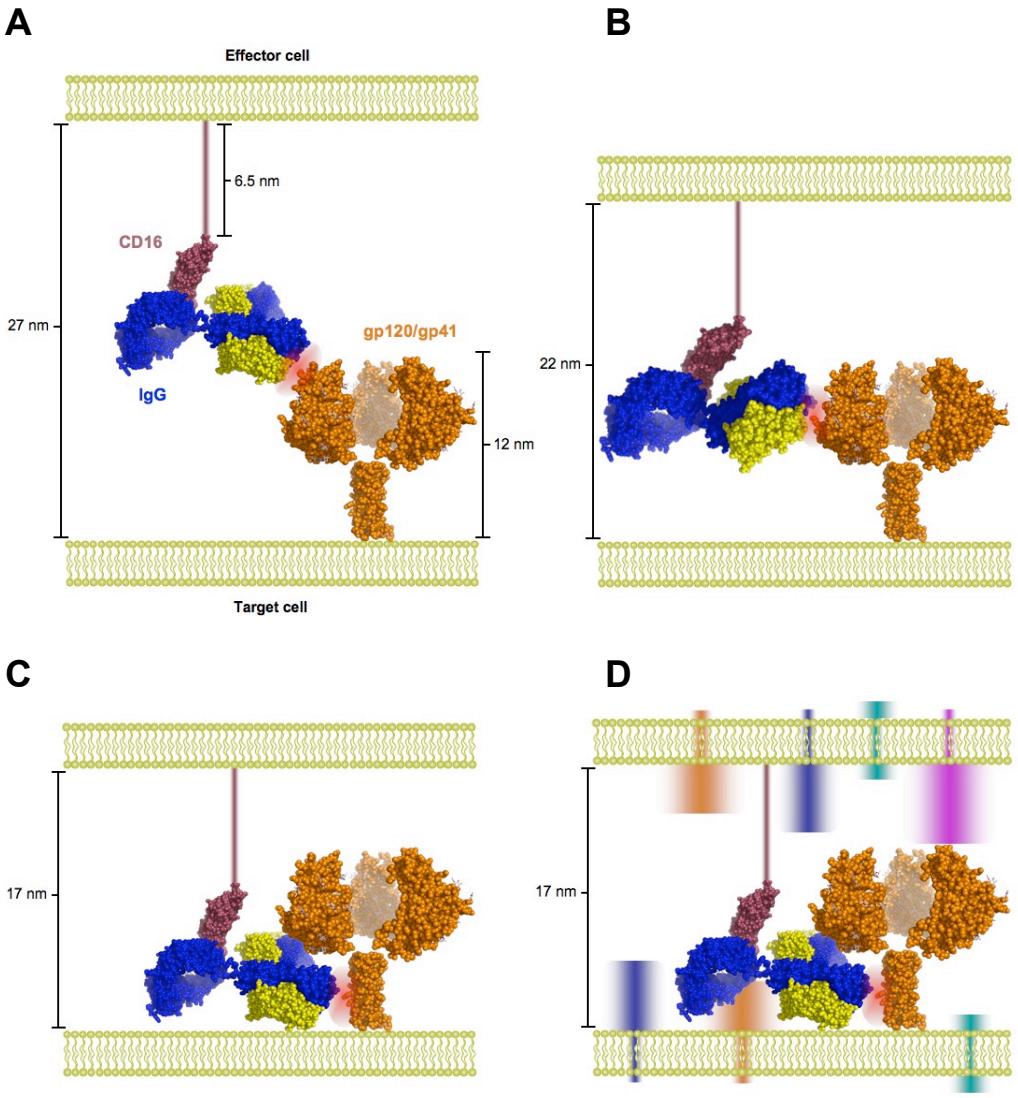

Figure 5. Conceptual model of the distance requirements for simultaneous binding of IgG1 with CD16 and the HIV envelope spike with the assumption that the 16-residue unstructured linker connecting the extracellular domains of CD16 to the membrane is adopting a fully extended state. The height for the envelope spike was based on reports of tomographic reconstructions of intact spikes (22). Schematics for antibody binding to (*A*) glycan clusters or the V3 loop region near the top of the spike, (*B*) the CD4 binding site, and (*C*) the MPER. (*D*) An illustration of the potential steric effects imposed by the presence of other membrane-bound proteins.

The architecture of IgG3 differs significantly from IgG1 in that it has an unusually long hinge region that serves to increase the distance between the Fc domain and the Fabs. Thus, it may be that the Fc domains of anti-gp120 antibodies are inherently more accessible to CD16a binding as compared to anti-MPER antibodies.

An additional explanation for the exceptional performance of the 2G12 antibodies in the ADCC assay relative to the antibodies that bind protein epitopes may be that multiple 2G12 antibodies are binding to each trimeric spike with avidity as a result of its high glycan content (see Chapter 3), thereby inducing localized high concentrations of bound CD16a on effector cells and amplifying the ADCC activation signal. For protein epitopes, there can exist no more than three binding sites per envelope spike, which cannot be cross-linked by a single antibody due to geometric constraints (see Fig. S3, Chapter 2). Consequently, the endocytosis signals in the cytoplasmic tail of gp41 that serve to limit the concentration of spikes on the surface of a cell (29, 30) may present a limit to highly avid binding by these antibodies and limit their capacity to cross-link CD16a.

The finding that CD16-binding was critical to the ability of IgG1 b12 to protect NHPs in the SHIV challenge model (12) highlights the possibility that future advancements in optimizing the therapeutic efficacy of anti-HIV antibodies may depend on their abilities to trigger ADCC. The clinical trials discussed in Chapter 3 where a cocktail composed of 2G12, 4E10, and 2F5 suppressed viral rebound among patients who underwent interruptions in HAART also demonstrated that escape mutations were found only for 2G12 and not 4E10 or 2F5 (31). Thus, it is tempting to speculate that the

selective pressure of 2G12 might have derived, at least in part, from an enhanced ability to trigger ADCC against infected cells.

Given the nearly universal failure to elicit potent neutralizing antibodies against HIV, one possible approach to increasing the effectiveness of the humoral immune response would be to develop a method to introduce engineered variants of anti-HIV antibodies with increased abilities to trigger ADCC by gene therapy. For example, Fc mutations have been identified that increase the affinity of the Fc for CD16a by greater than 2 orders of magnitude (32), resulting in a similar increase in ADCC activity *in vitro* as well as increased potency *in vivo*. As an additional approach, we are currently examining whether the RCEs described in Chapter 3 might exhibit increased ADCC activity as a result of increasing the hinge length.

Methods

Untransfected CHO-K1 cells were obtained from the American Type Culture Collection (ATCC) and maintained in R-10 media (RPMI-1640 supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% 5000 U/mL Pen/Strep, 1% NEAA, and 1% Na-pyruvate). The HxBc2 $gp160^{+}$ CHO cell line, derived from CHO-K1 cells and named CHO-WT (referred to as GHO-gp160 in this text) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from C. Weiss and J. White (14). These cells were maintained in G-MEM with no glutamine and supplemented with 10% dialyzed FCS, 3.7% $NaHCO_3$ at 75 mg/mL, 1% Pen/Strep at 5000 U/mL, 1% 100x non-essential amino acids, 1% 100x Na-pyruvate, 1% 100x glutamate/asparagine (each at 6 mg/mL), 2% 50x Nucleosides (3.5 mg/mL of cytidine,

uridine, guanosine, and adenosine; 1.2 mg/mL of thymidine), and 0.4% methionine sulfoximine (MSX) at 18 mg/mL (selection for glutamine synthetase transfectants).

PBMCs were purified from a lymphapheresis pack supplied by a single donor (Hemacare) as described in the manual (Ficoll Paque Plus User Manual, GE Healthcare). Briefly, donor blood was combined 1:1 with RPMI-1640 and 30 mL aliquots were layered onto 15 mL aliquots of Ficoll-Paque Plus in 50 mL conical tubes and centrifuged at 300 x g for 30 minutes at 4 °C. Buffy coats were then washed three times with RPMI 1640 and re-suspended in FCS to a concentration of 200 million cells per mL. 100 million cells were then combined 1:1 with freezing medium (20% DMSO, 80% FCS), frozen at -80 ºC, and stored in liquid nitrogen. PBMCs were reconstituted in R-10 media 24 to 48 hours prior to use in the assay.

Each antibody reagent was tested in triplicate. One million target cells were seeded into a six well plate and allowed to adhere overnight. The growth media were then replaced with identical media but lacking Glu/Met/Cys and supplemented with S^{35}-Met to 0.25 mCi. After incubation overnight at 37ºC, the cells were then detached with 2 mM EDTA in PBS, washed three times with R-10, and resuspended to a concentration of 200,000 cells per mL in R-10. Ten thousand target cells were then dispensed per well, to which 50 µL of antibody diluted into R-10 were added and incubated for 30 minutes on ice. PBMCs were washed once in R-10 and added to the appropriate wells to a final volume of 200 µL per well (100 µL/well for the maximum lysis condition). Plates were incubated for 4 hours at 37 ºC. Fifteen minutes prior to the end of the incubation period, 100 µL of 1 M NaOH was added to wells for the maximum lysis condition containing only labeled target cells. Plates were then centrifuged at 300 x g for 5 minutes and 50 µL

of each sample was transferred to white-bottomed Optiplate-96 reader plates already containing 200 μL of Microscint 40 scintillation fluid (PerkinElmer). Plates were then wrapped in foil and allowed to sit overnight at room temperature. Samples were read in a Wallac 1450 Microbeta TriLux. Specific lysis was calculated as $[(\alpha - \beta)/(\gamma - \beta)] \times 100\%$, where α is target cells with PBMCs and antibody, β is maximum lysis, and γ is target cells with PBMCs alone. The curves were fit with a four-parameter logistic equation (Prism 4 for Macintosh, GraphPad Software, Inc.).

The FITC-conjugated polyclonal goat anti-gp120 antibody was obtained from Abcam. Methods describing expression, purification, binding assays, neutralization assays, and molecular modeling can be found in Chapter 2. Rituxan was generously provided by Dr. Sanjeev Nandakumaran (Kaiser Permanente Southern California Medical Group).

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