Published as: *AIDS*. 2010 July 17; 24(11): 1633–1640.

A dimeric form of the HIV-1 antibody 2G12 elicits potent antibody-dependent cellular cytotoxicity

Joshua S. Klein[*,1], **Alexandre Webster**[*,1], **Priyanthi N. P. Gnanapragasam**[1], **Rachel P. Galimidi**[1], and **Pamela J. Bjorkman**[1,2,3]

[1]Division of Biology, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125

[2]Howard Hughes Medical Institute, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125

Abstract

Objective—Increasing data support a role for antibody-dependent cellular cytotoxicity (ADCC) in controlling HIV-1 infection. We recently isolated a naturally-occurring dimeric form of the anti-HIV-1 antibody 2G12 and found it to be significantly more potent than 2G12 monomer in neutralizing primary virus strains. However, given the unusual structure of dimeric 2G12 with two Fc regions, it was not clear whether 2G12 dimer could bind to the CD16 Fc receptor on ADCC effector cells or trigger ADCC. Here we compared the *in vitro* ADCC activities of 2G12 monomer and dimer and investigated the effects of including ADCC-enhancing mutations in both forms of 2G12.

Methods—An *in vitro* ADCC assay using target cells stably expressing gp160 was developed to evaluate the activities of 2G12 monomer and dimer with and without ADCC-enhancing mutations that increase the CD16-binding affinity of the 2G12 Fc region.

Results—Both 2G12 monomer and 2G12 dimer elicited ADCC, although the dimer showed increased potency (lower half-maximal concentration [EC_{50}]) in triggering ADCC, thus confirming its ability to bind CD16 and trigger ADCC. The ADCC-enhancing mutations improved the ADCC activity of 2G12 monomer more than 2G12 dimer such that their EC_{50} values were nearly equal. However, no increase in non-specific ADCC activity was observed using 2G12 IgGs with these mutations.

Conclusion—Given the likelihood that ADCC plays a role in protecting against initial infection and/or controlling chronic infection, these data suggest 2G12 dimers and/or addition of ADCC-enhancing mutations could augment the prophylactic and/or therapeutic potential of 2G12.

Keywords

antibodies; antibody-dependent cell cytotoxicity; HIV-1; immunotherapy; virus neutralization; 2G12

[3]Corresponding author. Mailing address: Division of Biology, California Institute of Technology, 1200 E. California Blvd., Pasadena, CA 91125. Phone: 626-395-8350. Fax: 626-395-5939. bjorkman@caltech.edu.
[*]These authors contributed equally to this work.

Introduction

Antibodies can prevent or contribute to the elimination of a viral infection by multiple mechanisms including neutralization (*i.e.*, binding directly to virus particles to prevent entry into target cells), antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis of infected cells upon opsonization, and activation of the classical pathway of complement [1]. ADCC is initiated by Fc receptors on innate immune cells recognizing the Fc regions of clustered IgG1 and IgG3 antibodies bound to the surface of infected cells, resulting in a cytolytic response against targeted cells [2]. Multiple subpopulations of peripheral blood mononuclear cells (PBMCs) are capable of ADCC [2, 3], but particular attention has been given to ADCC activity triggered by cross-linking CD16 (FcγRIIIa) on the surfaces of natural killer (NK) cells [4, 5], which are the largest subset of peripheral leukocytes after T and B cells [6].

Considerable evidence supports a role for ADCC activity in the control of human immunodeficiency virus type 1 (HIV-1) infection (reviewed in [1, 7]). HIV-infected individuals whose sera can trigger ADCC *in vitro* have lower viral titers and higher CD4+ T cell counts than infected individuals whose sera are less active in ADCC [8, 9]. ADCC-inducing antibodies arise earlier and in higher titers than neutralizing antibodies during the acute phase of HIV-1 infection [10–14]. The emergence of NK cell-dependent viral inhibition by IgG from HIV-1-infected donors is also correlated with reductions in viral load during the acute phase of infection [10], and the loss of CD16 expression on NK cells isolated during the chronic phase of infection is associated with increased viral load [15]. Lastly, when the ability to trigger ADCC through CD16 binding was eliminated for the broadly-neutralizing anti-HIV antibody b12, which targets the CD4 binding site of gp120, the mutant antibody could no longer protect macaques from viral challenge at dosages that were sufficient for protection by wild-type b12 [16]. Thus, while the ability to neutralize HIV-1 *in vitro* has generally received the greatest attention when judging the performance of anti-HIV antibodies [1], it is becoming increasingly clear that ADCC-mediated mechanisms of HIV-1 control deserve equally careful scrutiny.

2G12 is a potent neutralizing anti-HIV-1 IgG, which protects rhesus macaques against viral challenge [17, 18] and can trigger ADCC in an *in vitro* assay [19, 20]. 2G12 binds to a constellation of high mannose carbohydrates on the gp120 portion of the HIV-1 envelope spike using an unusual 3D domain swapped structure [21]. Typical IgGs contain two heavy chains and two light chains, which form two Fabs from the pairing of the light chain variable and constant domains (V_L and C_L) with the V_H and C_H1 domains of the heavy chain. In most IgGs, the Fabs are free to rotate independently about the hinge region that connects them to the Fc region, resulting in two antigen binding sites separated by distances ranging from 10 to 15 nm. However, domain-swapped monomeric IgG 2G12 has two V_H domains that have exchanged their V_L domain binding partners [21]. As a result, the two carbohydrate binding sites created by the V_H-V_L domains of the Fabs are separated by a fixed distance of ~3.5 nm, and two additional carbohydrate binding sites are created at the interface of the two V_H domains [21].

We recently reported that expression of 2G12 in mammalian cells results in a mixture of 2G12 monomer and a higher molecular weight oligomer that was characterized as a 2G12 dimer containing four heavy chains and four light chains, which combine to form four Fabs and two Fc regions [22]. The dimeric form of 2G12 exhibited 50- to 80-fold increased neutralization potency against a panel of clade A and B HIV-1 strains relative to the monomer [22]. We presented a model for how inter-domain swapping between two 2G12 monomers could create a 2G12 dimer [22], but the precise arrangement of the two Fc regions in dimeric 2G12 and the accessibility of its FcγR binding sites are unknown.

Here we investigated whether 2G12 dimer could elicit ADCC and whether the enhanced neutralization potency of dimeric versus monomeric 2G12 also extends to the FcγR-dependent function of ADCC. Previous results investigating ADCC induction by 2G12 monomers used an *in vitro* assay involving T cells coated with monomeric gp120 bound to CD4 as targets [19, 20]. Given that epitopes for monomeric and/or dimeric 2G12 might not be accurately preserved when using soluble monomeric gp120 bound to CD4, we developed an *in vitro* ADCC assay using a previously-described cell line stably transfected to express HXB2 gp160 [23] as target cells. We found that both monomeric and dimeric 2G12 elicited efficient ADCC responses with dimeric 2G12 being 6.8-fold more potent than monomeric 2G12. We also introduced mutations into the 2G12 Fc region that enhance its affinity for CD16 [24]. These mutations increased ADCC potency to a greater extent for 2G12 monomer as compared to 2G12 dimer, reducing their difference in potency to 3.3-fold. Given previous evidence for a significant protective effect by 2G12 in both human and primate studies [17, 25], these results suggest that incorporating ADCC-enhancing mutations into 2G12 and the use of dimeric 2G12 are worth consideration in the context of passively administered immunotherapy or gene therapy.

Materials and Methods

Protein expression and purification

IgG 2G12 ($2G12_{WT}$) was expressed transiently in suspension HEK 293-6E cells and purified from transfected cell supernatants using protein A chromatography as described [22]. Monomeric and dimeric versions of $2G12_{WT}$ in neutralized protein A eluates were separated using size exclusion chromatography by two or more passages over a Superdex 200 16/60 or 10/30 column (GE Healthcare, Piscataway, New Jersey, USA) [22]. Mutations that enhance the affinity of IgG (Ser239 to Asp and Ile332 to Glu) for CD16 and increase ADCC potency in the presence of effector cells [24] were introduced into the Fc region of the 2G12 heavy chain gene using the QuikChange mutagenesis kit (Agilent Technologies, La Jolla, California, USA) to create $2G12_{S239D/I332E}$. Monomeric and dimeric forms of $2G12_{S239D/I332E}$ were expressed and purified as described for $2G12_{WT}$. Purified rituximab (an IgG antibody against CD20 used as a negative control) was the gift of Dr. Sanjeev Nandakumaran (Kaiser Permanente Southern California Medical Group). All antibodies were of the human IgG1 subclass.

ADCC assay

Target cells were a Chinese hamster ovary cell line expressing the full-length envelope spike from the clade B strain HXB2 (CHO-WT; here referred to as CHO-gp160) and glutamine synthetase was used for selection and amplification [26], which was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CHO-SEC from Dr. Carol Weiss and Dr. Judith White. Another CHO cell line (CHO-HFE) [27], also generated using the glutamine synthetase system, was used as a negative control target cell line. Both cell lines were cultured in the absence of glutamine as previously described [23, 28].

Human peripheral blood mononuclear cells (PBMCs) were purified from a lymphophoresis pack obtained from a single healthy volunteer (Hemacare, Van Nuys, California, USA) by standard Ficoll-Hypaque gradient centrifugation (Ficoll-Paque PLUS User Manual, cat# 17-1440-02, GE Healthcare). Cells were immediately frozen at 100×10^6 cells/mL in 80% heat-inactivated fetal bovine serum (FBS) supplemented with 20% DMSO. Approximately 24 to 48 hours prior to use, cells were thawed and cultured in RPMI-complete media (RPMI1640 medium [Invitrogen, Carlsbad, California, USA] supplemented with 10% FBS, 2 mM glutamine [Invitrogen], 5000 U/mL Penicillin-Streptomycin (pen-strep) [Invitrogen],

1% MEM non-essential amino acids [Invitrogen], and 1% sodium pyruvate [Invitrogen]) at a density of 1.3×10^7 cells/mL in an upright 75 cm^2 flask to deplete adherent cells. ADCC assays were conducted by measuring radioactivity released from target cells metabolically-labeled with ^{35}S after incubation with IgG and purified PBMCs.

Target cells (CHO-gp160 or CHO-HFE) seeded into 96-well flat-bottom plates at 10,000 cells per well were labeled for 24 hours in cysteine, methionine and glutamine-free DMEM containing 250 μCi/mL ^{35}S EasyTag Express Protein Labeling Mix [Perkin Elmer, Waltham, Massachusetts, USA] and supplemented with 10% dialyzed, heat inactivated FBS, 200 μM methionine sulfoximine [Sigma-Aldrich, St. Louis, Missouri, USA], 3.7% $NaHCO_3$ [Sigma-Aldrich], 5000 U/mL pen-strep [Invitrogen], 1% MEM non-essential amino acids [Invitrogen], 1% sodium pyruvate [Invitrogen], 0.007% cytidine, uridine, guanosine, and adenosine [Sigma-Aldrich], 0.006% asparagine and glutamate [Sigma-Aldrich]. Cells were washed gently five times in PBS followed by reconstitution in RPMI-complete media and then incubated on ice for 15 minutes. Antibody reagents were tested for ADCC activity in triplicate at ten concentrations of a three-fold dilution series, starting with 150 μg/mL and 16.7 μg/mL, respectively, for monomeric and dimeric IgG species. PBMCs were added at an effector to target cell ratio of 50:1 and incubated at 37°C for 4 hours. Once terminated, 50 μL of reaction supernatant was added to 150 μL Microscint 40 scintillation fluid [PerkinElmer] and incubated for 16–18 hours in the dark at room temperature in Optiplate-96 microplates [PerkinElmer] followed by liquid scintillation counting using a MicroBeta TriLux scintillation counter [PerkinElmer]. In some experiments, cells were labeled in batch in a 6-well plate at 500,000 cells per well and harvested with 5 mM EDTA after 24 hours. After washing five times in PBS, cells were reconstituted with RPMI-complete media into a 96-well round-bottom plate at 10,000 cells per well prior to incubation with IgG and PBMCs. No significant differences in EC_{50} values were observed between the two approaches.

Rituximab (150 μg/mL) was tested in parallel as an isotype control for non-specific lysis against CHO-gp160 and CHO-HFE with and without PBMCs. The level of spontaneous ^{35}S release was determined by incubating target cells in RPMI-complete media alone, while the levels of non-specific PBMC-associated ^{35}S release and non-antibody-associated ^{35}S release were respectively obtained by incubating target cells with PBMCs or antibody alone for the duration of the assay. Maximal ^{35}S release levels were obtained by incubating target cells in a final concentration of 750 mM NaOH 15 minutes prior to the termination of the ADCC assay. Percentage maximal lysis was determined as follows: (^{35}S release with antibody and PBMC – ^{35}S release with PBMC)/(maximal ^{35}S release – ^{35}S release with PBMC) × 100%. Curves were fit using Prism version 4.0a (Graphpad Software, San Diego, California, USA) to the equation $L=N+(M-N)/\{1+10^{[(LogEC50-X)*H]}\}$ where L is percentage lysis, M is the maximal percentage lysis, N is the minimal percentage lysis, X is the Log of the concentration of reagent being tested, and H is the Hill coefficient. We observed decreasing levels of maximal lysis at concentrations greater than 50 μg/mL and 5.5 μg/mL for monomer and dimer, respectively. This effect has been observed in ADCC studies unrelated to HIV and is likely attributable to CD16 saturation by IgG not bound to target cells (J. Vielmetter, personal communication); thus, these points were omitted from the curve fitting. Variations in lysis maxima were observed in some experiments; however, variation in EC_{50} values between replicates for any particular reagent remained low regardless of the amount of maximal lysis.

In vitro neutralization assay

Pseudovirus neutralization assays were used to compare the neutralization potencies of monomeric and dimeric forms of $2G12_{WT}$ and $2G12_{S239D/I332E}$ against HXB2 pseudoviruses. The assay, which measures the reduction in luciferase reporter gene

expression in the presence of a potential inhibitor following a single round of pseudovirus infection in TZM-bl cells [29, 30], was conducted using minor modifications as described [31, 32]. Neutralization data were fit by non-linear regression analysis using Prism version 4.0a (Graphpad Software) and IC_{50} values were derived as described [31]. Tests of statistical significance for the neutralization and ADCC assays were done using the two-tailed Student's t-test.

Results

Development of an ADCC assay using target cells expressing HIV-1 spike trimers

Multiple conserved N-linked glycans on the surface of gp120 have been identified that contribute to the ability of 2G12 to bind to and neutralize HIV [20, 33, 34]. In particular, structure-based analysis of mutagenesis data was used to identify several glycosylation sites near the coreceptor binding site as being particularly critical components of the 2G12 epitope [34], and their elimination has been linked to escape from 2G12 *in vivo* [17, 35]. However, questions still remain with regard to the nature of 2G12 binding that could undermine the previously-described method of using CD4+ cells coated with monomeric gp120 as targets for ADCC [19, 20]. For example, the epitope(s) of 2G12 monomers and/or dimers may span between gp120 subunits in a trimer. In addition, the presentation and accessibility of FcγR-binding sites on 2G12 may differ when bound to a native trimeric spike versus monomeric gp120 bound to CD4, and multiple binding orientations may exist for monomeric and/or dimeric 2G12 on an intact trimer. Lastly, effects on ADCC potency arising from the potential for multiple 2G12 antibodies to be bound to a single trimeric spike would not be accurately replicated when using monomeric gp120. For these reasons, we developed an ADCC assay using target cells previously shown to stably express trimeric gp160 [23]. In addition to resolving the concerns outlined above, gp160-expressing target cells offer additional advantages over cells coated with monomeric gp120 in that they can be used to test the abilities of antibodies to trigger ADCC for epitopes that overlap the CD4 binding site of gp120, target the gp41 domain, and/or require a non-CD4 bound conformation of gp120.

Conditions for the ADCC assay included a 15-minute pre-incubation period of antibody with target cells on ice followed by the addition of human PBMCs at an effector-to-target cell ratio of 50:1 and incubation for 4 hours at 37 °C. Low background lysis was observed with the addition of a monoclonal anti-CD20 isotype control antibody, rituximab, to either the CHO-HFE control or the CHO-gp160 cells and for all 2G12 antibodies against the CHO-HFE cells (Fig. 1A), indicating that 2G12 is specific for gp120 despite its anti-carbohydrate reactivity. The lack of ADCC activity by 2G12 against the control target cells is consistent with a previous study that observed no significant lysis of CD4+ T cells in the presence of 2G12 and human PBMCs [20]. Maximal lysis against CHO-gp160 target cells was variable between independent replicates for all of the reagents tested. However, the difference between the average maximal lysis for any two reagents was not statistically significant ($p > 0.05$ for all pairwise combinations).

IgG 2G12 elicits more potent ADCC as a dimer than monomer

We recently described isolation of 2G12 monomers and dimers that do not detectably interconvert and reported that purified dimeric 2G12 was an average of 50- to 80-fold more potent than monomeric 2G12 in the neutralization of pseudotyped primary virus isolates from clades A and B [22]. Here we confirmed the higher neutralization potency of $2G12_{WT}$ dimer versus $2G12_{WT}$ monomer against the HXB2 HIV-1 strain (Fig. 1B) in order to make comparisons with the HXB2-expressing target cells used in ADCC assays (CHO-gp160 cells). The HXB2 strain is unusually sensitive to neutralization by 2G12 monomer [22];

thus, the increase in potency for 2G12 dimer as compared to the monomer was less than observed in comparisons using less-sensitive primary virus isolates [22].

To investigate whether the enhanced neutralization potency of dimeric $2G12_{WT}$ over monomeric $2G12_{WT}$ also extended to the ability to elicit ADCC, we compared their abilities to trigger ADCC *in vitro*. We found that both monomeric and dimeric $2G12_{WT}$ elicited efficient ADCC responses with half-maximal effective concentration (EC_{50}) values of 2.3 ± 0.8 and 0.34 ± 0.22 μg/mL, respectively (Table 1, Fig. 1C, D). Thus, dimeric $2G12_{WT}$ exhibited an average 6.8-fold increased potency as compared to monomeric $2G12_{WT}$ ($p < 0.001$).

Fc mutations increase the ADCC potency of both monomeric and dimeric 2G12

Although the increased potency of dimeric versus monomeric $2G12_{WT}$ observed in the neutralization assay also extended to ADCC activity, the absolute values for the potencies of both forms were 5- to 8-fold weaker in the ADCC assay, such that the average concentrations at which half maximal neutralization was achieved for $2G12_{WT}$ monomer and dimer were 0.42 μg/mL and 0.044 μg/mL, respectively, versus average concentrations at which half maximal lysis by ADCC was achieved for monomer and dimer, which were 2.3 μg/mL and 0.34 μg/mL, respectively. The higher concentrations of $2G12_{WT}$ required for ADCC as compared to concentrations required for neutralization could result from the low affinity of IgG Fc regions for CD16 [24, 38].

To investigate the effects of increasing the affinity for CD16, we introduced a double mutation to the Fc of 2G12 (S239D and I332E) that was previously shown to enhance the affinity of IgG for CD16 and increase ADCC potency [24]. The mutant protein, $2G12_{S239D/I332E}$, was expressed and monomeric and dimeric forms were purified. The mutations introduced to the 2G12 Fc region did not adversely affect the neutralization potencies of either the monomeric or dimeric proteins: The IC_{50} values for $2G12_{S239D/I332E}$ monomer and dimer were 0.14 μg/ml and 0.031 μg/ml, respectively, as compared with average IC_{50} values for $2G12_{WT}$ monomer and dimer of 0.42 ± 0.11 μg/ml and 0.044 ± 0.013 μg/ml, respectively (Table 1). When evaluated in the ADCC assay, the ADCC-enhancing mutations did not result in increased background activity against the CHO-HFE control target cells (Fig. 1A), but we observed increased ADCC potency for both $2G12_{S239D/I332E}$ monomer and $2G12_{S239D/I332E}$ dimer against the target cells, yielding EC_{50} values of 0.17 ± 0.15 and 0.051 ± 0.035 μg/mL, respectively (Table 1, Fig. 1C, D). While the increase in potency for the $2G12_{S239D/I332E}$ monomer versus the $2G12_{WT}$ monomer was statistically significant ($p < 0.001$), the difference in potency between the two dimeric forms was not statistically significant ($p = 0.07$). These results suggest that $2G12_{S239D/I332E}$ and $2G12_{WT}$ dimers bind CD16 with similar affinities, likely because avidity effects arising from the presence of two Fc regions would limit the potential affinity enhancement of the Fc mutations.

Discussion

Here we compared the abilities of monomeric 2G12 and a recently described naturally-occurring dimeric form of 2G12 [22] to trigger ADCC by human PBMCs against target cells stably expressing gp160. The increased potency of dimeric 2G12 over its monomeric form in the neutralization of HIV-1 [22] was observed to also extend to its ADCC activity, confirming the ability of Fc receptors on effector cells to bind one or both Fc regions on dimeric 2G12. An ADCC-enhancing double mutation (Ser239 to Asp; Ile332 to Glu) that increases the affinity of the Fc region of IgG for the activating receptor CD16 [24] yielded a greater decrease in the EC_{50} value for monomeric $2G12_{S239D/I332E}$ as compared to dimeric $2G12_{S239D/I332E}$ in the ADCC assay. As a result, both forms of $2G12_{S239D/I332E}$ showed

similar potencies. Importantly, these ADCC-enhancing mutations did not increase non-specific ADCC activity against target cells that did not express gp160 (Fig. 1A).

Because 2G12 dimer has twice as many Fabs and Fc regions as the monomeric form, the 6.8-fold increase in potency (i.e., 6.8-fold lower EC_{50} value) observed for $2G12_{WT}$ dimer as compared to $2G12_{WT}$ monomer could be attributed to an increased affinity for gp160, an increased affinity for Fc receptors on the effector cells, or both. Given that the similarity in EC_{50} values observed for $2G12_{S239D/I332E}$ monomer and dimer was driven mostly by the increased potency of the monomeric form (Table 1), we conclude that the lower potency observed for $2G12_{WT}$ monomer can primarily be attributed to the weak ability of monomeric wild-type IgG Fc regions in eliciting ADCC via CD16 engagement [24, 38].

Several lines of evidence suggest that further evaluations of the *in vivo* properties, particularly the potential for eliciting ADCC, of potential anti-HIV antibodies would be valuable. First, eliminating ADCC activity but not complement activity severely reduced the ability of IgG b12 to protect rhesus macaques from vaginal challenge with chimeric simian/human immunodeficiency virus (SHIV) [16]. Additional evidence is related to early events in vaginal transmission of SIV in rhesus macaques, where it has been shown that the virus quickly crosses the epithelial barrier to infect a small founder population of cells in the endocervical region [39–41], and a local expansion of this founder population may be providing a necessary supply of infected cells that migrate to the lymphatic tissues to fuel a systemic infection (reviewed in [42]). Given the pivotal role that the founder population of infected cells may play in establishing a productive systemic infection, containment or the prevention of its establishment altogether represent critical opportunities for intervention in the transmission process [42]. Unfortunately, the window of opportunity to accomplish either of these objectives appears to be narrow: only hours for prevention after exposure and approximately 3 days for containment [39, 40]. As NK cells are capable of mediating ADCC without pre-stimulation by the adaptive immune system, a gene therapy that could deliver a continuous supply of a neutralizing antibody that is also highly active in ADCC would be well-suited to targeting both prevention and containment of the founder population.

Evidence specifically in support of the use of 2G12 as a potential therapeutic may be derived from a comparison of the *in vivo* anti-viral and pharmokinetic properties of 2G12 with b12 and two other broadly neutralizing antibodies, 4E10 and 2F5. For example, studies have shown that 2G12 can protect against challenge by SHIV in rhesus macaques at substantially lower serum neutralizing titers than observed for b12 [17, 18, 43]. In addition, a study of human HIV-positive subjects who were injected with a cocktail of 2G12, 4E10, and 2F5 indicated that of the three IgGs, only 2G12 exerted selective pressure against infection. In this study, administration of the antibody cocktail resulted in a significant delay in viral rebound after cessation of anti-retroviral therapy [25], but sequence analysis after eventual viral rebound confirmed the emergence of escape mutations at conserved glycosylation sites that abrogated sensitivity to 2G12, whereas no escape mutations were found to 4E10 or 2F5 [35]. The same study also found that 2G12 exhibited the longest half-life of the three antibodies. Our current finding that 2G12 dimers retain the ability to elicit ADCC, along with our recent finding that $2G12_{WT}$ dimer exhibits significantly increased potency and expanded cross-reactivity relative to the monomer [22], suggest that $2G12_{WT}$ dimer and $2G12_{S239D/I332E}$ monomer and dimer are promising candidates for use in passive immunotherapy or prophylactic gene therapy.

Acknowledgments

We thank Drs. Jost Vielmetter and Tal Arnon for advice about the ADCC assay, Christopher Foglesong and the Caltech Protein Expression Center for expressing 2G12, Dr. Sanjeev Nandakumaran (Kaiser Permanente Southern California Medical Group) for purified rituximab, and Kathryn Huey-Tubman, Matt Thornton, Anthony West and

Fan Yang for critical reading of the manuscript. This work was supported by the Bill and Melinda Gates Foundation through the Grand Challenges in Global Health Initiative, the Collaboration for AIDS Vaccine Discovery, the Howard Hughes Medical Institute, and an NIH National Research Service Award 5 T32 GM07616 (A.W).

Funding sources: Bill and Melinda Gates Foundation (BMGF) through the Grand Challenges in Global Health Initiative; Collaboration for AIDS Vaccine Discovery (CAVD); Howard Hughes Medical Institute (HHMI); National Institutes of Health National Research Service Award (NIH NRSA).

References

1. Huber M, Trkola A. Humoral immunity to HIV-1: neutralization and beyond. J Intern Med. 2007; 262:5–25. [PubMed: 17598812]
2. van de Winkel JG, Capel PJ. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. Immunol Today. 1993; 14:215–221. [PubMed: 8517920]
3. Daeron M. Fc receptor biology. Annu Rev Immunol. 1997; 15:203–234. [PubMed: 9143687]
4. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001; 22:633–640. [PubMed: 11698225]
5. Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. What is a natural killer cell? Nat Immunol. 2002; 3:6–8. [PubMed: 11753399]
6. Freud AG, Caligiuri MA. Human natural killer cell development. Immunol Rev. 2006; 214:56–72. [PubMed: 17100876]
7. Iannello A, Debbeche O, Samarani S, Ahmad A. Antiviral NK cell responses in HIV infection: I. NK cell receptor genes as determinants of HIV resistance and progression to AIDS. J Leukoc Biol. 2008; 84:1–26. [PubMed: 18388298]
8. Ahmad R, Sindhu ST, Toma E, Morisset R, Vincelette J, Menezes J, Ahmad A. Evidence for a correlation between antibody-dependent cellular cytotoxicity-mediating anti-HIV-1 antibodies and prognostic predictors of HIV infection. J Clin Immunol. 2001; 21:227–233. [PubMed: 11403230]
9. Forthal DN, Landucci G, Keenan B. Relationship between antibody-dependent cellular cytotoxicity, plasma HIV type 1 RNA, and CD4+ lymphocyte count. AIDS Res Hum Retroviruses. 2001; 17:553–561. [PubMed: 11350669]
10. Forthal DN, Landucci G, Daar ES. Antibody from patients with acute human immunodeficiency virus (HIV) infection inhibits primary strains of HIV type 1 in the presence of natural-killer effector cells. J Virol. 2001; 75:6953–6961. [PubMed: 11435575]
11. Bottiger B, Ljunggren K, Karlsson A, Krohn K, Fenyo EM, Jondal M, Biberfeld G. Neutralizing antibodies in relation to antibody-dependent cellular cytotoxicity-inducing antibodies against human immunodeficiency virus type I. Clin Exp Immunol. 1988; 73:339–342. [PubMed: 3208445]
12. Aasa-Chapman MM, Hayman A, Newton P, Cornforth D, Williams I, Borrow P, et al. Development of the antibody response in acute HIV-1 infection. Aids. 2004; 18:371–381. [PubMed: 15090788]
13. Sawyer LA, Katzenstein DA, Hendry RM, Boone EJ, Vujcic LK, Williams CC, et al. Possible beneficial effects of neutralizing antibodies and antibody-dependent, cell-mediated cytotoxicity in human immunodeficiency virus infection. AIDS Res Hum Retroviruses. 1990; 6:341–356. [PubMed: 1971182]
14. Nixon DF, Broliden K, Ogg G, Broliden PA. Cellular and humoral antigenic epitopes in HIV and SIV. Immunology. 1992; 76:515–534. [PubMed: 1383131]
15. Liu Q, Sun Y, Rihn S, Nolting A, Tsoukas PN, Jost S, et al. Matrix metalloprotease inhibitors restore impaired NK cell-mediated antibody-dependent cellular cytotoxicity in human immunodeficiency virus type 1 infection. J Virol. 2009; 83:8705–8712. [PubMed: 19553339]
16. Hessell AJ, Hangartner L, Hunter M, Havenith CE, Beurskens FJ, Bakker JM, et al. Fc receptor but not complement binding is important in antibody protection against HIV. Nature. 2007; 449:101–104. [PubMed: 17805298]
17. Hessell AJ, Rakasz EG, Poignard P, Hangartner L, Landucci G, Forthal DN, et al. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV

challenge even at low serum neutralizing titers. PLoS Pathog. 2009; 5:e1000433. [PubMed: 19436712]

18. Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med. 2000; 6:207–210. [PubMed: 10655111]
19. Gomez-Roman VR, Florese RH, Patterson LJ, Peng B, Venzon D, Aldrich K, Robert-Guroff M. A simplified method for the rapid fluorometric assessment of antibody-dependent cell-mediated cytotoxicity. J Immunol Methods. 2006; 308:53–67. [PubMed: 16343526]
20. Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, et al. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol. 1996; 70:1100–1108. [PubMed: 8551569]
21. Calarese DA, Scanlan CN, Zwick MB, Deechongkit S, Mimura Y, Kunert R, et al. Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. Science. 2003; 300:2065–2071. [PubMed: 12829775]
22. West AP Jr, Galimidi RP, Foglesong CP, Gnanapragasam PN, Huey-Tubman KE, Klein JS, et al. Design and expression of a dimeric form of human immunodeficiency virus type 1 antibody 2G12 with increased neutralization potency. J Virol. 2009; 83:98–104. [PubMed: 18945777]
23. Weiss CD, White JM. Characterization of stable Chinese hamster ovary cells expressing wild-type, secreted, and glycosylphosphatidylinositol-anchored human immunodeficiency virus type 1 envelope glycoprotein. J Virol. 1993; 67:7060–7066. [PubMed: 8230430]
24. Lazar GA, Dang W, Karki S, Vafa O, Peng JS, Hyun L, et al. Engineered antibody Fc variants with enhanced effector function. Proc Natl Acad Sci U S A. 2006; 103:4005–4010. [PubMed: 16537476]
25. Trkola A, Kuster H, Rusert P, Joos B, Fischer M, Leemann C, et al. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat Med. 2005; 11:615–622. [PubMed: 15880120]
26. Bebbington, CR.; Hentschel, CCG. The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells. In: Glover, DM., editor. DNA cloning : Vol. III, A Practical Approach. IRL Press, Ltd.; 1987. p. 163-188.
27. Lebron JA, Bennett MJ, Vaughn DE, Chirino AJ, Snow PM, Mintier GA, et al. Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. Cell. 1998; 93:111–123. [PubMed: 9546397]
28. Vile, R. Selectable Markers for Eukaryotic Cells. In: Collins, MK., editor. Practical Molecular Virology: Viral Vectors for Gene Expression. Clifton: Humana Press Inc.; 1991. p. 49-60.
29. Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, et al. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J Virol. 2005; 79:10108–10125. [PubMed: 16051804]
30. Montefiori DC. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Curr Protoc Immunol. 2005; Chapter 12 Unit 12 11.
31. Klein JS, Gnanapragasam PN, Galimidi RP, Foglesong CP, West AP Jr, Bjorkman PJ. Examination of the contributions of size and avidity to the neutralization mechanisms of the anti-HIV antibodies b12 and 4E10. Proc Natl Acad Sci U S A. 2009; 106:7385–7390. [PubMed: 19372381]
32. West AP Jr, Galimidi RP, Foglesong CP, Gnanapragasam PN, Klein JS, Bjorkman PJ. Evaluation of CD4-CD4i Antibody Architectures Yields Potent, Broadly Cross-Reactive Anti-Human Immunodeficiency Virus Reagents. J Virol. 2010; 84:261–269. [PubMed: 19864392]
33. Scanlan CN, Pantophlet R, Wormald MR, Ollmann Saphire E, Stanfield R, Wilson IA, et al. The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120. J Virol. 2002; 76:7306–7321. [PubMed: 12072529]
34. Sanders RW, Venturi M, Schiffner L, Kalyanaraman R, Katinger H, Lloyd KO, et al. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J Virol. 2002; 76:7293–7305. [PubMed: 12072528]

35. Manrique A, Rusert P, Joos B, Fischer M, Kuster H, Leemann C, et al. In vivo and in vitro escape from neutralizing antibodies 2G12, 2F5, and 4E10. J Virol. 2007; 81:8793–8808. [PubMed: 17567707]

36. Alsmadi O, Herz R, Murphy E, Pinter A, Tilley SA. A novel antibody-dependent cellular cytotoxicity epitope in gp120 is identified by two monoclonal antibodies isolated from a long-term survivor of human immunodeficiency virus type 1 infection. J Virol. 1997; 71:925–933. [PubMed: 8995609]

37. Koup RA, Robinson JE, Nguyen QV, Pikora CA, Blais B, Roskey A, et al. Antibody-dependent cell-mediated cytotoxicity directed by a human monoclonal antibody reactive with gp120 of HIV-1. Aids. 1991; 5:1309–1314. [PubMed: 1722676]

38. Okazaki A, Shoji-Hosaka E, Nakamura K, Wakitani M, Uchida K, Kakita S, et al. Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcgammaRIIIa. J Mol Biol. 2004; 336:1239–1249. [PubMed: 15037082]

39. Zhang Z, Schuler T, Zupancic M, Wietgrefe S, Staskus KA, Reimann KA, et al. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science. 1999; 286:1353–1357. [PubMed: 10558989]

40. Miller CJ, Li Q, Abel K, Kim EY, Ma ZM, Wietgrefe S, et al. Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. J Virol. 2005; 79:9217–9227. [PubMed: 15994816]

41. Hu J, Gardner MB, Miller CJ. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. J Virol. 2000; 74:6087–6095. [PubMed: 10846092]

42. Haase AT. Targeting early infection to prevent HIV-1 mucosal transmission. Nature. 464:217–223.

43. Parren PW, Marx PA, Hessell AJ, Luckay A, Harouse J, Cheng-Mayer C, et al. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J Virol. 2001; 75:8340–8347. [PubMed: 11483779]

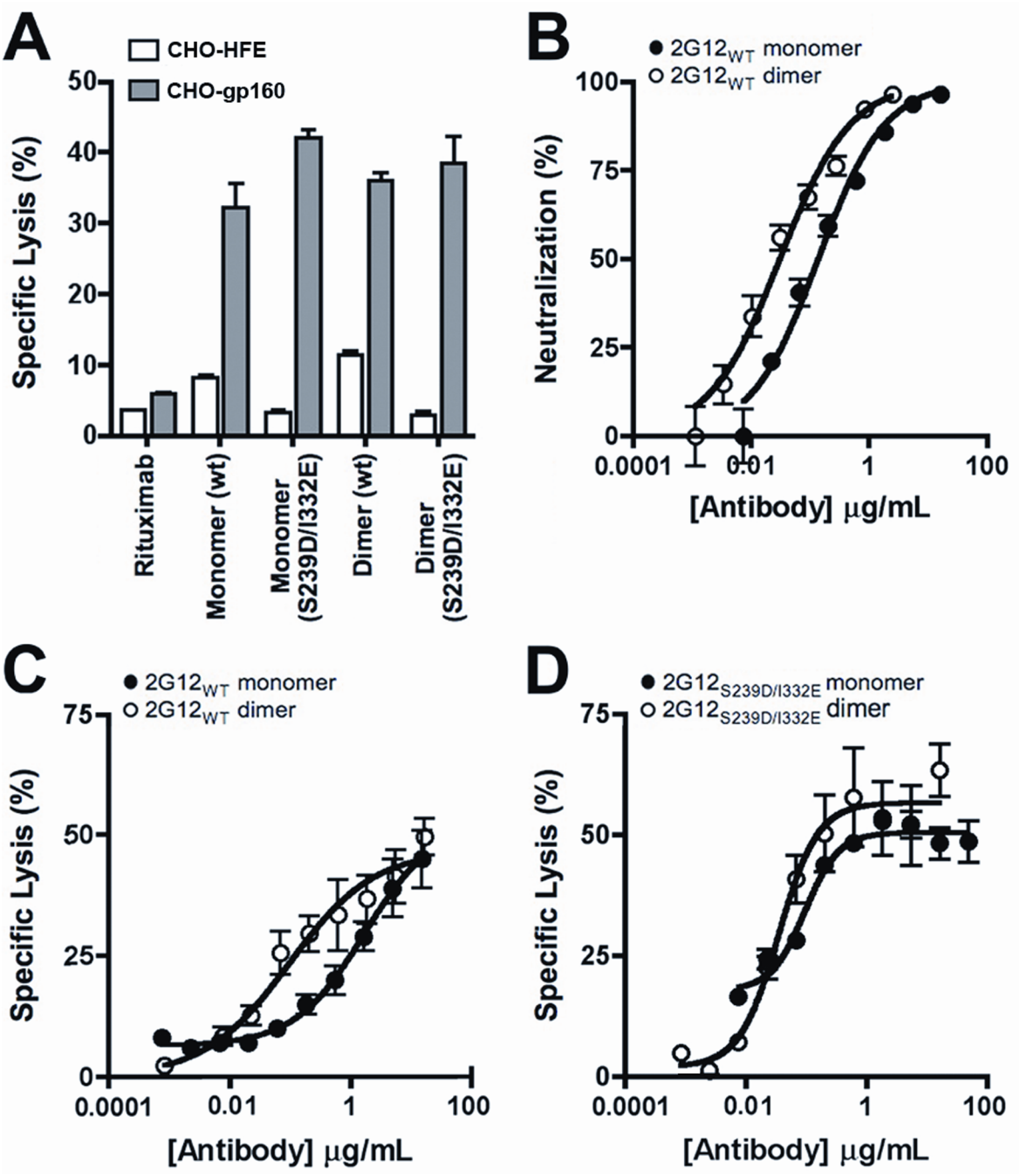

Figure 1.

ADCC and neutralization activity of 2G12. (A) Comparison of maximal lysis of target cells expressing HXB2 trimers (CHO-gp160) and control cells (CHO-HFE) after addition of rituximab (150 μg/mL), $2G12_{WT}$ monomer (150 μg/mL), $2G12_{S239D/I332E}$ monomer (150 μg/mL), $2G12_{WT}$ dimer (17 μg/mL), and $2G12_{S239D/I332E}$ dimer (17 μg/mL). Error bars are the standard errors from a representative experiment conducted in triplicate. (B) Representative neutralization assays to compare potencies of $2G12_{WT}$ monomer and $2G12_{WT}$ dimer against HXB2 HIV-1. Data points are the average of a triplicate measurement ± the standard error of the mean. Average IC_{50} values determined from independent neutralization assays, each conducted using triplicate measurements, are

reported in Table 1. (C–D) Representative ADCC assays using CHO-gp160 target cells by monomeric and dimeric $2G12_{WT}$ (panel C) or monomeric and dimeric $2G12_{S239D/I332E}$ (panel D). Data points in the curves are the average of a triplicate measurement ± the standard error of the mean. Average EC_{50} values determined from curves from independent ADCC assays, each conducted using triplicate measurements, are reported in Table 1.

Table 1

Comparison of average half-maximal effective concentrations (EC_{50}s) in ADCC assays and half-maximal neutralization concentrations (IC_{50}s) in pseudovirus-based neutralization assays for monomeric and dimeric 2G12 against strain HXB2. Values are reported as the average ± the standard deviation from n independent replicates. Bracketed values represent the average ± the standard deviation in maximal lysis. Representative ADCC and neutralization curves are shown in Fig. 1.

	EC_{50} (μg/mL)		IC_{50} (μg/mL)	
Form of 2G12	**$2G12_{WT}$**	**$2G12_{S239D/I332E}$**	**$2G12_{WT}$**	**$2G12_{S239D/I332E}$**
monomer	2.3 ± 0.8 (n = 6) [44 ± 27%][c]	0.17 ± 0.15 (n = 5)[a] [55 ± 31%][c]	0.42 ± 0.11 (n = 5)	0.14 (n = 1)
dimer	0.34 ± 0.22 (n = 5)[a] [33 ± 9%][c]	0.051 ± 0.035 (n = 3)[a] [47 ± 8%][c]	0.044 ± 0.013 (n = 5)[b]	0.031 (n = 1)

[a] $p < 0.005$ when compared to the average EC_{50} value for $2G12_{WT}$ monomer.

[b] $p < 0.0001$ when compared to the average IC_{50} value for $2G12_{WT}$ monomer.

[c] $p > 0.05$ for all pair-wise combinations.