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A Nonfucosylated Variant of the anti-HIV-1 Monoclonal Antibody b12 Has Enhanced FcγRIIIa-Mediated Antiviral Activity *In Vitro* but Does Not Improve Protection against Mucosal SHIV Challenge in Macaques

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Eliciting neutralizing antibodies is thought to be a key activity of a vaccine against human immunodeficiency virus (HIV). However, a number of studies have suggested that in addition to neutralization, interaction of IgG with Fc gamma receptors (FcγR) may play an important role in antibody-mediated protection. We have previously obtained evidence that the protective activity of the broadly neutralizing human IgG1 anti-HIV monoclonal antibody (MAb) b12 in macaques is diminished in the absence of FcγR binding capacity. To investigate antibody-dependent cellular cytotoxicity (ADCC) as a contributor to FcγR-associated protection, we developed a nonfucosylated variant of b12 (NFb12). We showed that, compared to fully fucosylated (referred to as wild-type in the text) b12, NFb12 had higher affinity for human and rhesus macaque FcγRIIIa and was more efficient in inhibiting viral replication and more effective in killing HIV-infected cells in an ADCC assay. Despite these more potent *in vitro* antiviral activities, NFb12 did not enhance protection *in vivo* against repeated low-dose vaginal challenge in the simian-human immunodeficiency virus (SHIV)/macaque model compared to wild-type b12. No difference in protection, viral load, or infection susceptibility was observed between animals given NFb12 and those given fully fucosylated b12, indicating that FcγR-mediated activities distinct from FcγRIIIa-mediated ADCC may be important in the observed protection against SHIV challenge.

Numerous studies indicate a role for the extraneutralizing functions of human immunodeficiency virus (HIV) antibodies in protection against the virus (3, 4, 13, 15, 19, 21, 22, 24, 28, 38, 43). Of these functions, antibody-dependent cellular cytotoxicity (ADCC) is often suggested to be a key mechanism (2, 7, 17). In support, findings using rhesus macaques have provided evidence that protection against simian-human immunodeficiency virus (SHIV) and simian immunodeficiency virus (SIV) challenge can be influenced by antibody interaction with FcγRs (13, 19, 21, 22). Specifically, we have previously obtained evidence that a broadly neutralizing human IgG1 b12 antibody deficient in Fcγ receptor (FcγR) binding (LALA) has diminished protective capacity relative to wild-type b12 *in vivo* (21, 22). In addition, immunization studies have suggested that vaccine-induced elicitation of ADCC-mediating antibodies correlates with reduced SIV viremia (13, 19), and, in elite controllers, ADCC-specific antibodies have been suggested to contribute to the control of viral load (28). Natural killer (NK) cells are one of the major effector cells for ADCC and are recruited through binding of the cellular leukocyte receptor FcγRIIIa to the Fc part of the IgG antibody (11). Human IgG Fc CH2 domains each contain a single *N*-linked oligosaccharide consisting of a conserved core structure containing three mannose and two *N*-acetylglucosamine residues, optional core fucose and *N*-acetylglucosamine residues, and optional galactose and sialic acid residues attached to arms emerging from the core. Fc glycosylation heavily impacts FcγR binding capacity and antibody effector functions. IgG molecules with Fc glycan lacking the core fucose residue display an increased affinity for FcγRIIIa and enhanced ADCC (12, 40, 41), and nonfucosylated versions of human antibodies with enhanced ADCC have been used in the treatment of cancer (6, 23, 41, 44, 49).

To investigate the role of FcγRIIIa-mediated ADCC in protection of rhesus macaques against SHIV, we generated a nonfucosylated b12 (NFb12) antibody. We showed that nonfucosylated IgG b12 antibody has higher affinity for FcγRIIIa, as well as enhanced antibody-dependent cell-mediated virus inhibition

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(ADCVI), ADCC and NK cell activation potency, than the wild-type b12 antibody. However, using the low-dose repeated-challenge model, no enhanced protection against SHIV was achieved by treating rhesus macaques with NFb12 compared to wild-type antibody b12.

MATERIALS AND METHODS

Macaques. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Wisconsin (protocol number g00575). All antibody infusions, viral challenges, and sample collections were performed under ketamine- or ketamine-medetomidine-induced anesthesia, and all efforts were made to minimize suffering. At the start of the experiments, all animals were experimentally naive and were negative for antibodies against HIV-1, SIV, and type D retrovirus. Antibody administration and viral challenges followed a weekly schedule; antibody (1 mg/kg) was administered intravenously (i.v.) on Thursdays, and viral intravaginal (i.vag.) challenges (30 50% tissue culture infective doses [30 $TCID_{50}$]) were administered on Fridays and Mondays. Protocols are more fully described elsewhere (22, 34). All animals were given 30 mg of medroxyprogesterone (Depo-Provera) intramuscularly (i.m.) at days −28 and 0 and then every 22 to 28 days until the first sign of viremia.

Challenge virus. The challenge virus was $SHIV_{SF162}$ passage 3 virus (20, 45) and was propagated in phytohemagglutinin-activated rhesus macaque peripheral blood mononuclear cells (PBMCs). The original stock was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (catalog number 6526; contributors, J. Harouse, C. Cheng-Mayer, and R. Pal).

b12, NFb12, and control antibodies. IgG1 b12 is a human antibody [IgG1(κ)] that recognizes an epitope overlapping the CD4 binding site of gp120 (5). Recombinant IgGs (wild-type b12, LALA, and DEN3) were expressed in Chinese hamster ovary (CHO-K1) cells as previously described (22, 34). LALA is a b12 variant deficient in binding to all Fcγ receptors (21). We used DEN3, a dengue virus-specific NS1 human IgG1 antibody, as the isotype control antibody in this study. NFb12 was generated by subcloning PCR-amplified V_L and V_H fragments of the b12 antibody into the pKANTEX93 vector (31). The antibody expression vector was transfected by electroporation into a FUT8 knockout CHO cell line (49). Cells were selected in hypoxanthine-thymidine (HT)-free medium containing 200 nM methotrexate (MTX), and single cells were isolated. A high-producing clone was selected, and culture supernatant was subjected to antibody purification by protein A column chromatography. Antibodies used for the passive transfer experiments contained less than 1 unit of endotoxin per mg. The oligosaccharide profile of purified NFb12 was characterized by modified matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) with a positive ion mode as described previously (33).

Recombinant human and rhesus macaque FcγRs. Generation of human His-tagged FcγRI, FcγRIIa-H131, FcγRIIIa-F158, and FcγRIIIa-V158 extracellular domains was performed as previously described (35). Generation of rhesus macaque His-tagged FcγRIIIa-1 (or FcγRIIIa-I212) (the extracellular soluble molecule FcγRIIIa-1 is identical in sequence to FcγRIIIa-2; these two molecules differ in the transmembrane/intracellular region that is not expressed in the soluble molecule) and FcγRIIIa-3 (or FcγRIIIa-V212) was performed as described elsewhere (S. L. Finstad et al., unpublished data). Briefly, plasmids encoding the extracellular region of rhesus macaque FcγRIIIa (RMFcγRIIIa) variants were transfected into HEK293T/17 cells (CRL-11268; ATCC). Proteins were purified from cell culture supernatant using a HisPur Cobalt resin purification kit according to the manufacturer's specifications (Thermo Scientific, Waltham, MA). Proteins were subsequently dialyzed in 1× phosphate-buffered saline (PBS) and concentrated. Protein concentrations were determined by the Bradford assay.

ELISA. We determined b12 and NFb12 antibody binding as well as concentrations in macaque serum by enzyme-linked immunosorbent assay (ELISA) using recombinant monomeric HIV-1 SF162 gp120, as described elsewhere for HIV-1 JRFL gp120 (34). FcγR-specific (except for rhesus macaque FcγRIIa) ELISAs were performed by coating ELISA plates with a concentration of 5 μg/ml of b12, NFb12, or LALA. Plates were blocked with 5% milk before incubation with 3-fold dilutions of His-tagged FcγR protein. Horseradish peroxidase (HRP)-conjugated mouse anti-polyhistidine antibody (MAB050H; R&D Systems, Minneapolis, MN) was used as a detection conjugate, and binding was visualized using 3,3′,5,5′-tetramethylbenzidine (TMB) as the HRP substrate. Rhesus macaque FcγRIIa-specific ELISAs were performed by coating the ELISA plates with Fc receptor at 5 μg/ml. Plates were blocked with 3% bovine serum albumin (BSA) before incubation with 5-fold dilutions of wild-type b12, NFb12, or LALA. Binding was detected with HRP-conjugated anti-human $F(ab)_2$ antibody (Jackson ImmunoResearch, West Grove, PA), and binding was visualized using TMB as the HRP substrate. Anti-human antibodies in macaque plasma were determined by coating ELISA plates with b12 or NFb12 at 0.2 μg/ml. Plates were blocked with 3% BSA before incubation with 20-fold or 100-fold dilutions of plasma samples. Binding of anti-human antibodies to b12 and NFb12 was determined by subsequent incubations with biotinylated b12 (or NFb12) at 0.4 μg/ml and streptavidin-alkaline phosphatase ([AP] Jackson ImmunoResearch laboratories, West Grove, PA). Binding was visualized using a phosphatase substrate (Sigma-Aldrich, St. Louis, MO). Biotinylation of antibodies was done using an EZ-Link Micro Sulfo-NHS-LC-biotin (sulfosuccinimidyl-6-biotinamido-hexanoate) kit (Therma Scientific, Rockford, IL) following the manufacturer's guidelines. To minimize interference by administered antibodies, plasma samples were obtained approximately 1 month after the last antibody administration.

Cell surface binding assays. The cell surface binding assay was performed as described elsewhere (47). Briefly, 293T cells were cotransfected with HIV-1 Env-expressing plasmid (pSVIII-JRFL) and pSG3ΔEnv. Transfected cells were stained with serial dilutions of wild-type b12, NFb12, or DEN3. Binding of antibodies was detected using goat anti-human IgG F(ab′) conjugated to phycoerythrin (Jackson ImmunoResearch laboratories, West Grove, PA). Stained cells were analyzed using an Accuri C6.

SPR measurements. Surface plasmon resonance (SPR) measurements were performed using a Biacore 2000. Antibodies (500 resonance units [RU]) were amine coupled onto a CM5 biosensor chip (Biacore, Piscataway, NJ). FcγRs (2 μM; 2-fold serial dilutions, with nine dilutions in total) were injected over the antibody surface at 30 μl/min for 3 min, followed by an 8-min dissociation phase. Sensorgrams were analyzed with Scrubber2, version 2.0a (BioLogic Software Pty. Ltd., Australia). Background binding obtained by injection of FcγRs over a JRCSF gp120-coupled surface as well as injection of running buffer only was subtracted from the experiment sensorgrams. Equilibrium dissociation constants (K_Ds) were determined from steady-state binding values.

Antibody concentration in vaginal secretion. Antibody concentrations in mucosal secretions were determined as previously described (34). Briefly, vaginal secretions from each animal were absorbed to cellulose wicks (Solan Weck-Cel surgical spears; Xomed Surgical Products, Jacksonville, FL). Three samples per animal were taken at 4, 6, 8, 12, 24, 48, 72, 96, and 168 h after i.v. administration of 5 mg/kg of body weight of antibody. Supernatant was extracted from the wicks and used to determine the concentration of antibody in mucosal secretions by ELISA.

Neutralization. Replication-incompetent HIV-1 enveloped pseudovirus was generated by cotransfection of 293T cells with HIV-1 Env-expressing plasmid (pSVIII) and pSG3ΔEnv as previously described (29). Serial dilutions of wild-type b12, NFb12, DEN3, or serum samples were preincubated with pseudovirus for 1 h at 37°C before being added to TZM-bl cells. Luciferase reporter gene expression was evaluated at 2 days

postinfection. The antibody and serum dilution producing a 50% inhibitory concentration (IC_{50}) was calculated by regression analysis using GraphPad Prism.

ADCVI. The ADCVI assay was based on a method previously described (16). Briefly, target cells (CEM.NKR-CCR5 cells infected with $SHIV_{SF162P3}$ for 48 h) were incubated with serial dilutions of monoclonal antibody (MAb) and effector cells (human PBMCs or human monocytes) at a ratio of 1 to 10 (PBMCs) or 1 to 1 (monocytes). Seven days later, p27 from the supernatant was determined by ELISA (Zeptometrix Corporation, Buffalo, NY). Virus inhibition at each concentration of MAb was determined in comparison to a negative-control MAb (DEN3). CEM.NKR-CCR5 cells were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (catalog number 4376; contributor, Alexandra Trkola).

ADCC. Target cells (NKr.CEM-CCR5 cells containing a Tat-inducible luciferase gene) were infected with $SHIV_{SF162P3}$ 4 days prior to use. An NK cell line (derived from KHYG-1 cells [48]) (Japan Health Sciences Foundation, Japan) stably expressing human FcγRIIIa-V158 was used as an effector. Effector and target cells were incubated at a 10:1 ratio in the presence of serial dilutions of antibodies. After 8 h, luciferase activity was measured using BriteLite Plus luciferase substrate (Perkin Elmer, San Jose, CA). The luciferase signal in wells containing effectors and uninfected targets was defined as 0% relative light units (RLU), whereas wells containing effectors and infected targets were defined as 100% RLU.

NK degranulation assay. Human NK degranulation was assessed by flow cytometry, modified from a previously described method (1). Autologous CD4 cells (target cells) and NK cells (effector cells) were used to eliminate the possibility of non-self-recognition. Activated CD4 cells were infected with vesicular stomatitis virus (VSV)-pseudotyped full-length HIV JRCSF for 2 days (50 to 80% infection on day 2). The cells were cocultured at an effector-to-target ratio of 1:1 in the presence of 20 μl/ml CD107a-phycoerythrin (PE)-Cy5, 0.3 μg/ml GolgiStop (BD Biosciences, La Jolla, CA), and 0.5 μg/ml of brefeldin A (Sigma, St. Louis, MO) in complete RPMI medium. Cells were cocultured for 6 h before being stained with CD3-Pacific Blue, CD56-PE-Cy7, CD16-allophycocyanin (APC)-Cy7, and CD4-Amcyan (BD Biosciences) to define the effector and target cell subsets. The cells were washed and fixed before measurement on a BD LSRII instrument. Data were analyzed using FlowJo (Tree Star, Ashland, OR). Background levels of NK cell activation by HIV-infected autologous cells were subtracted from experimental values, and a response was considered positive if the frequency of CD107a-expressing cells was at least 3-fold greater than 3 standard deviations over the mean of unstimulated NK cells. The 50% effective concentration (EC_{50}) for wild-type b12 was estimated by extrapolating the curve to the same level (75% activation) as the maximum level observed for NFb12.

Plasma viral loads. The quantity of SHIV viral RNA (vRNA) genomic copy equivalents (vRNA copy Eq/ml) in EDTA-anticoagulated plasma was determined using a quantitative reverse-transcription PCR (QRT-PCR) assay (10). Briefly, vRNA was isolated from plasma, and QRT-PCR was performed using a SuperScript III Platinum One-Step Quantitative RT-PR System (Invitrogen, Carlsbad, CA). Reactions were run on a Roche LightCycler, version 2.0, instrument, and vRNA copy number was determined using LightCycler, version 4.0, software (Roche Molecular Diagnostics, Indianapolis, IN) to interpolate sample crossing points onto an internal standard curve prepared from 10-fold serial dilutions of a synthetic RNA transcript representing a conserved region of SIV Gag.

FcγR genotyping. The genotypes for FcγRIIa and FcγRIIIa were determined for all animals. Allele sequences were obtained by performing sequence-specific PCR amplification for both genes using peripheral blood leukocyte (PBL)-derived cDNA, as described elsewhere (Finstad et al., unpublished).

MHC genotyping. Major histocompatibility complex (MHC) genotyping by sequence-specific PCR was performed by the University of Wisconsin Genotyping Core as previously described (22).

Statistical analyses. The animal experiment consisted of a total of 14 animals ($n = 14$) divided into three groups: four control animals ($n = 4$), five animals ($n = 5$) treated with wild-type b12, and five animals ($n = 5$) treated with NFb12. Statistical analyses were carried out using GraphPad Prism for Mac, version 5.0a (Graph Pad). A Kaplan-Meier survival analysis was performed for the data shown in Fig. 5. Hazard ratios (Cox proportional hazard model and Wald chi-square test) were calculated using SAS, version 9.2 (SAS, Cary, NC). Tests were not adjusted for multiple testing, and the *P* values (alpha level of 0.05) should be interpreted accordingly.

RESULTS

Generation of nonfucosylated b12. To investigate whether FcγRIIIa-mediated ADCC can contribute to *in vivo* protection of rhesus macaques against SHIV, we generated a nonfucosylated version of IgG1 b12 by stable transfection of a previously developed FUT8-deficient CHO cell line with an IgG1 b12-expressing plasmid. Nonfucosylated antibodies have been shown in cancer research to mediate notably enhanced ADCC relative to wild-type antibodies (6, 23, 44). FUT8 catalyzes the transfer of fucose to N-linked oligosaccharides, and antibodies generated in these cells therefore lack fucose (5, 49). A MALDI-TOF-MS analysis showed no evidence of fucose for the NFb12 antibody compared to 94% fucose for wild-type b12 generated in FUT8-proficient CHO cells.

Binding to human and rhesus macaque FcγRs. To evaluate the affinity of NFb12 for FcγRIIIa, we performed surface plasmon resonance (SPR) and ELISA studies comparing the interactions of NFb12 and wild-type b12 with recombinant human (genotypes F158 and V158) and rhesus macaque I212 (genotypes 1 or 2) and V212 (genotype 3) FcγRIIIa. NFb12 showed increased affinity to all FcγRIIIa proteins (6- to 8-fold by SPR) compared to wild-type b12 (Fig. 1; see also Fig. S1A in the supplemental material). Binding to the other activating Fc gamma receptors was largely unaffected by the absence of fucose (see Fig. S1B and C). As expected from previous studies (14, 39, 41), these experiments demonstrate that IgG fucosylation largely impacts binding to FcγRIIIa exclusively, with NFb12 showing a substantially increased affinity for this receptor.

***In vitro* antiviral activities of NFb12.** As expected, the antigen specificity, binding to cell surface-expressed envelope, and neutralization (HIV strains JRFL and JRCSF were tested in addition [data not shown]) properties of NFb12 were equivalent to those of wild-type b12 (Fig. 2A, B, and C). We next evaluated NFb12 in a series of Fc-dependent effector function assays for which activity of wild-type b12 could be shown (see Discussion below). In an antibody-dependent cell-mediated viral inhibition (ADCVI) assay (16) using a $SHIV_{SF162P3}$-infected human T-cell line as target cells, NFb12 gave a 10-fold increase in viral inhibition for human PBMCs (Fig. 3A) as effector cells relative to b12 but no increase for human monocytes (Fig. 3B) as effector cells in a comparable experiment. As freshly isolated monocytes mainly express FcγRIIa (9), these results nicely demonstrate the importance of FcγRIIIa and NK cells in the enhanced ADCVI activity of NFb12 compared to wild-type b12. We next performed an ADCC assay using engineered human NK cells as effector cells and showed that NFb12 mediated killing of HIV-infected target cells 10-fold more efficiently than wild-type b12, as seen by a decrease in luciferase signal from target cells (Fig. 3C). Finally, we performed an NK cell degranulation assay (1, 8), and, as seen in Fig. 3D, NFb12 possessed an increased ability to induce NK degranulation compared to

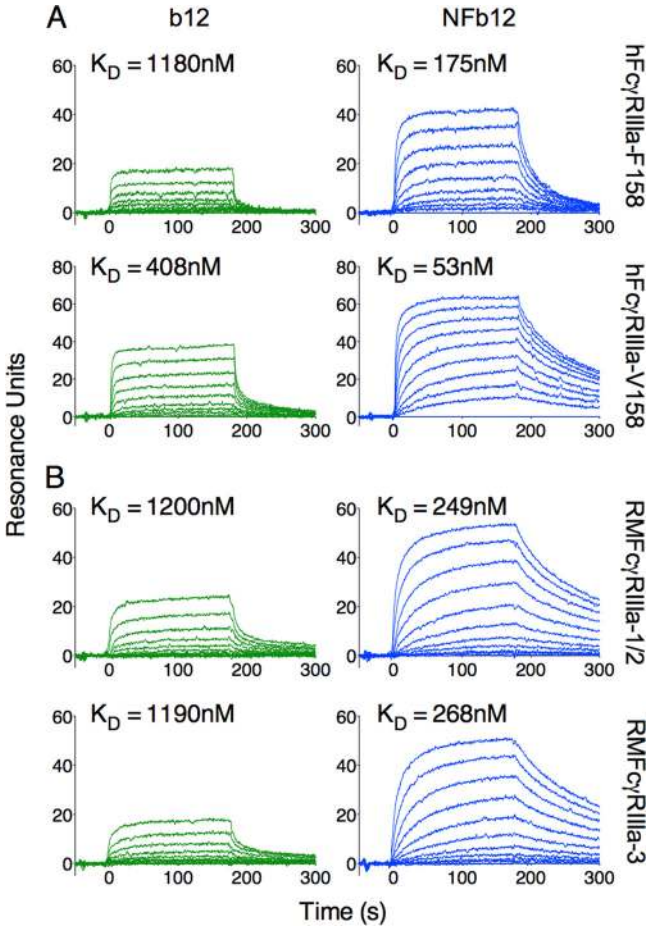

FIG 1 Binding of wild-type b12 and NFb12 to human and rhesus macaque FcγRIIIa. Binding of wild-type b12 and NFb12 to human FcγRIIIa (hFcγRIIIa) F158 and V158 (A) and rhesus macaque FcγRIIIa-1 and -2 (RMFcγRIIIa-1/2) and RMFcγRIIIa-3 (B) was evaluated by SPR. NFb12 showed an increase in affinity (K_D values) for allelic variants of human and rhesus macaque FcγRIIIa compared to wild-type b12. The assays were performed twice with similar results.

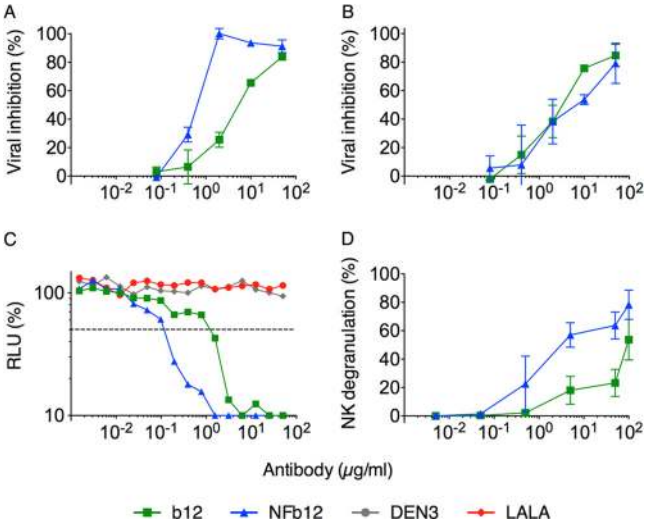

FIG 3 *In vitro* Fc-dependent activities of wild-type b12 and NFb12. (A and B) ADCVI assay. Target cells were infected with $SHIV_{SF162P3}$ for 48 h before incubation with effector cells and antibodies. Viral inhibition was measured after 7 days by a p27-specific ELISA. (A) NFb12 showed a 10-fold enhanced potency in viral inhibition compared to wild-type b12 in the presence of PBMCs (IC_{50} for b12, 4.37 μg/ml; IC_{50} for NFb12, 0.43 μg/ml). Values are mean and standard deviations ($n = 2$). (B) Wild-type b12 and NFb12 showed comparable potencies in viral inhibition in the presence of monocytes (IC_{50} for b12, 3.97 μg/ml; IC_{50} for NFb12, 6.97 μg/ml). Values are mean and standard deviations ($n = 2$). (C) ADCC assay. Killing of target cells is seen as a decrease in luciferase activity (RLU). NFb12 showed 10-fold enhanced killing of target cells compared to wild-type b12 (IC_{50} for b12, 1.3 μg/ml; IC_{50} for NFb12, 0.13 μg/ml). LALA and DEN3 did not mediate killing of infected cells as LALA did not recruit effector cells and DEN3 did not recognize HIV gp120. Values are the average of two independent experiments done in triplicate. (D) NK degranulation assay. NK degranulation was measured by CD107a expression. NFb12 shows a 40-fold enhanced ability to activate (degranulate) NK cells compared to wild-type b12 (estimated EC_{50} for b12, 58 μg/ml; EC_{50} for NFb12, 1.4 μg/ml). Values are mean and standard deviations ($n = 3$). LALA is a b12 variant deficient in binding to Fcγ receptors. DEN3 is a negative IgG1 control anti-dengue virus antibody.

wild-type b12. Wild-type b12 did not reach saturation, but extrapolating the curve to reach the maximum level of NFb12 showed that NFb12 activated NK cells 40-fold more efficiently than wild-type b12. Overall, the effector function assays clearly demonstrate that the *in vitro* antiviral FcγRIIIa-dependent activity of NFb12 is superior to that of wild-type b12.

Pharmacokinetics of NFb12. The *in vivo* properties of NFb12 were first evaluated in a pharmacokinetics study. Removal of fucose has not been reported to influence binding to the neonatal Fc

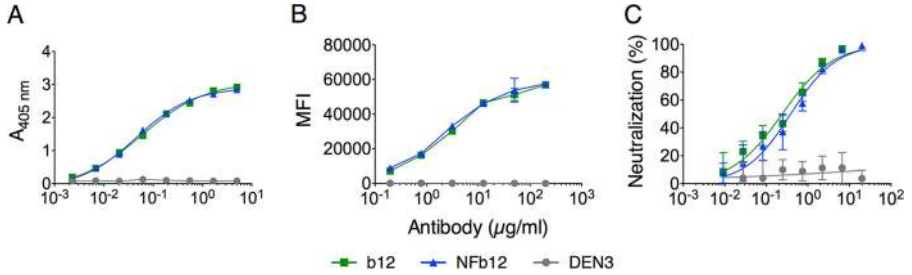

FIG 2 *In vitro* Fab-dependent activities of wild-type b12 and NFb12. (A) HIV SF162 gp120 ELISA. Wild-type b12 and NFb12 bound HIV gp120 with similar apparent affinities. Values are mean and standard deviations ($n = 2$). (B) Cell surface binding of wild-type b12 and NFb12 to HIV JRFL envelope. Wild-type b12 and NFb12 bound cell surface-expressed HIV JRFL envelope with comparable potencies. Binding curves were generated by plotting the mean fluorescence intensity (MFI) of antigen binding as a function of antibody concentration. Values are mean and standard deviations ($n = 2$). (C) Single-round $SHIV_{SF162P3}$ pseudovirus neutralization assay. Wild-type b12 and NFb12 neutralized $SHIV_{SF162P3}$ pseudovirus with comparable potencies (IC_{50} for b12, 0.24 μg/ml; IC_{50} for NFb12, 0.38 μg/ml). Control IgG1 antibody DEN3 did not neutralize virus. Values are mean and standard deviations ($n = 6$).

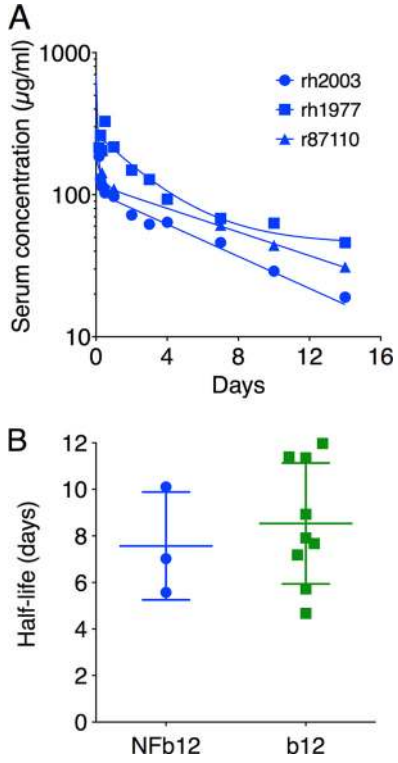

FIG 4 NFb12 and b12 half-lives in rhesus macaque. (A) Three animals were administered 5 mg/kg NFb12 i.v., and serum levels were determined by ELISA. Half-lives were calculated using a two-phase decay model. (B) No difference was observed in half-lives between wild-type b12 (8.5 days) and NFb12 (7.5 days) ($P = 0.48$, Mann-Whitney test). b12 values were adapted from Hessell et al. (21).

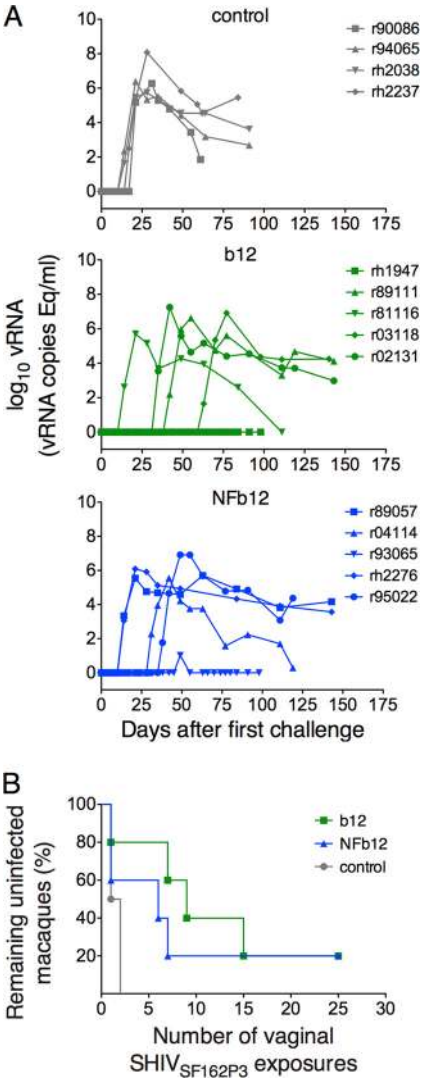

FIG 5 Protection of wild-type b12- and NFb12-treated rhesus macaques in a low-dose repeated $SHIV_{SF162P3}$ challenge experiment. (A) Viral loads for antibody-treated (1 mg/kg) rhesus macaques in a low-dose (30 $TCID_{50}$) repeated SHIV challenge study. Four animals were used as controls; two were untreated, and two were treated with isotype control antibody DEN3: five animals were treated with wild-type b12, and five animals were treated with NFb12. All animals in the control group became infected within two challenges whereas one animal in the b12 group and NFb12 group remained uninfected after 25 challenges (see Table S1 in the supplemental material). The minimum detection level was 150 SHIV RNA copies/ml (2.1 $\log_{10}$ vRNA copies/ml) with a 95% confidence level. (B) Adapted Kaplan-Meier analysis. The percentage of uninfected animals was plotted as a function of the number of viral challenges. The curve for the b12 group was significantly different from that of the control group ($P = 0.0361$). Although there was a clear trend for better protection offered by NFb12 relative to the control antibody, this did not reach statistical significance ($P = 0.1502$) in this analysis. There is no statistical difference in protection between wild-type b12 and NFb12 ($P = 0.5152$) (Mantel-Cox log rank test).

receptor (41) (responsible for recycling internalized IgG back to the cell surface), so similar half-lives for NFb12 and wild-type b12 were expected and, indeed, seen (Fig. 4). In addition, measuring wild-type b12 and NFb12 concentrations in the vaginal compartment following i.v. administration showed comparable levels of transudation through the mucosal tissue (as similar concentrations were achieved in mucosal secretions) (see Fig. S2 in the supplemental material).

Repeated low-dose challenge study in rhesus macaques. To better mimic viral doses that may be encountered in most human exposures, we used the low-dose repeated-challenge model (25, 26, 32). In our previous low-dose study (22), we used a challenge dose of 10 $TCID_{50}$ of $SHIV_{SF162P3}$; however, a cumulative 108 challenges resulted in infection of only four out of five animals treated with wild-type b12. In the present study, our main hypothesis was to investigate whether NFb12 had an increased protective effect compared to wild-type b12. However, given that wild-type b12 protected so effectively against a challenge dose of 10 $TCID_{50}$ of $SHIV_{SF162P3}$, here we decided to increase the challenge dose 3-fold to 30 $TCID_{50}$ of $SHIV_{SF162P3}$. The experiment included 14 rhesus macaques: four control animals (two untreated and two treated with isotype control antibody), five treated with wild-type b12, and five treated with NFb12. All animals were given medroxyprogesterone (Depo-Provera), as described previously (34), to synchronize menstrual cycles and thin the vaginal mucosa. To maintain serum concentrations of antibody, the animals were given weekly i.v. treatments of 1 mg/kg control antibody, wild-type b12, or NFb12. The animals were intravaginally challenged twice weekly (at 24 h and 96 h after antibody administration) with 30 $TCID_{50}$ of $SHIV_{SF162P3}$. Table S1 in the supplemental material summarizes antibody treatments, viral challenges, day of detection of viremia, day of peak viremia for each animal, and development of anti-human antibodies.

TABLE 1 Relative risk of infection as determined by the Cox proportional hazard model[a]

Groups compared	Hazard ratio	95% CI
b12 vs control	4.7	0.85–26.32
NFb12 vs control	3.0	0.59–14.74

[a] The hazard ratios for b12 and NFb12 show that the risk of infection at each challenge was reduced by the factors shown. The hazard ratios for b12 ($P = 0.0765$) and NFb12 ($P = 0.1853$) are not significantly different from the control (Wald chi-square test). CI, confidence interval.

TABLE 2 Statistical comparison of infection susceptibilities of different treatment groups[a]

Group	No. of challenges leading to infection	No. of challenges not leading to infection
Control	4	2
b12	4	48
NFb12	4	31

[a] A reduction in infection susceptibility was shown when the total number of challenges resulting in infection was compared to the total number of challenges not leading to infection. b12 ($P = 0.0022$) and NFb12 ($P = 0.0086$) are significantly different from the control. b12 and NFb12 ($P = 0.7089$) are not significantly different from each other (Fisher's exact test).

All control animals were infected within two viral challenges, whereas four of the animals treated with wild-type b12 became virus positive after 1, 7, 9, or 15 viral challenges, respectively, and the fifth macaque remained uninfected after 25 challenges (Fig. 5A; see also Table S1 in the supplemental material). Four of the NFb12-treated animals became virus positive after 1, 1, 6, or 7 viral challenges, respectively, and, as in the b12-treated group, the fifth macaque remained uninfected after 25 challenges (Fig. 5A; see also Table S1).

NFb12 does not enhance protection compared to wild-type b12. We used three methods to compare the levels of protection in the different groups. First, an adapted Kaplan-Meier analysis showed that passively transferred b12 offered significantly better protection than the control antibody ($P = 0.0361$). However, although there was a clear trend for better protection conferred by NFb12 relative to the control antibody, this did not reach statistical significance ($P = 0.1502$) (Fig. 5B). No statistical difference in protection between wild-type b12 and NFb12 was observed ($P = 0.5152$) (Fig. 5B). Second, using a Cox proportional hazard model, we calculated the relative risk of infection for wild-type b12 and NFb12 compared to the control group and found that wild-type b12 reduced the infection risk per challenge by a factor of 4.7 whereas NFb12 reduced it by a factor of 3.0 (Table 1). However, although clear trends were apparent, the hazard ratios did not reach statistical significance. The corresponding *P* values were as follows: for b12 versus control, $P = 0.0765$; NFb12 versus control, $P = 0.1853$; and b12 versus NFb12, $P = 0.5154$. Interestingly, the hazard ratio for wild-type b12 versus control antibody was notably lower here (4.7) than in a previous study (21.3) (22). This correlates with the higher challenge dose used here (30 $TCID_{50}$) than in previous challenge (10 $TCID_{50}$), suggesting that the hazard ratio is highly sensitive to the viral challenge dose, which may be an important consideration in evaluating the potential of antibody in mediating protection under different exposure conditions. Third, the infection susceptibility (37) was calculated for each treatment group to show that both wild-type b12-treated ($P = 0.0022$) and NFb12-treated ($P = 0.0086$) animals required a significantly higher number of challenges to become infected than control animals (Table 2). No difference was observed between wild-type b12- and NFb12-treated animals ($P = 0.7089$) (Table 2). Overall, our analyses strongly suggest that weekly treatment with a low dose of wild-type b12 or NFb12 induces an increase in the level of protection against viral challenge *in vivo*. However, there was no indication that NFb12 enhanced protection relative to wild-type b12.

Serum antibody concentration and neutralization titer. Serum levels of the transferred antibodies were monitored throughout the study. Of note, one b12-treated animal (r81116) achieved only minimal serum antibody concentrations for unknown reasons (see Fig. S3 and Table S2 in the supplemental material). However, no statistically significant difference between the remaining b12-treated animals and the NFb12-treated animals ($P = 0.064$) could be demonstrated although we did observe a trend toward a slightly higher serum concentration of NFb12 at the time of infection (Table 3). The repeated administration of human antibodies could potentially induce an immune response against the antibody and result in rapid clearing. To investigate whether any difference could be observed between NFb12 and wild-type b12, we carried out an ELISA-based test of plasma samples. All animals were negative for anti-human antibodies except rh2131 (b12-treated) and r95022 (NFb12-treated), which showed a weak response against the b12 antibodies. Serum neutralizing titers were also determined at the estimated time of infection, and no differences in IC_{50}s ($P = 0.548$) or 90% inhibitory concentrations ([IC_{90}s] $P = 0.917$) were observed between b12- and NFb12-treated animals (Table 3; see also Table S2). Despite the presence of anti-human antibodies in the sera of rh2131 and r95022, the

TABLE 3 Average serum neutralization titer and antibody concentration

Group and animal	Avg IC_{50}[a,c]	Avg IC_{90}[a,c]	Avg serum Ab concn[b,c]
b12- treated group			
r89111	28.00	2.050	13.50
r81116	37.00	3.600	5.75[d]
r03118	28.75	1.575	19.25
r02131	16.25	2.675	15.50
rh1947	23.50	3.300	13.25
NFb12- treated group			
r89057	27.75	1.650	20.50
r04114	17.75	1.575	15.75
rh2276	18.25	3.150	17.25
r93065	41.25	6.300	23.75
r95022	15.00	3.350	26.50

[a] Numbers represent average IC_{50} and IC_{90} values of day 0 and 3 time points before detection of viremia or termination of the experiment (see Table S2 in the supplemental material for details).
[b] Numbers represent average serum antibody (Ab) concentration of day 0 and 3 time points before detection of viremia or termination of experiment (see table S2 for details).
[c] No significant difference between average IC_{50} ($P = 0.548$), average IC_{90} ($P = 0.917$), and average serum antibody ($P = 0.064$) was observed between b12- and NFb12-treated animals (two-tailed Mann Whitney test).
[d] b12-treated r81116 achieved only minimal serum antibody concentrations for unknown reasons and was excluded from this analysis. When r81116 was included, a significant difference was reached between average serum antibody level of b12- and NFb12-treated animals ($P = 0.032$).

serum antibody concentrations and neutralizing titers for these animals were within the range seen for the other animals in the study (see Fig. S3 and Table S2).

Fcγ receptor and MHC genotyping. Sequencing of Fcγ receptors and MHCs showed uniform allele frequency distribution throughout the groups. It is worth noting that animal r90086 expressed Mamu-B*08 and that animal r81116 expressed Mamu-B*08 and Mamu-B*17, which likely contributed to the control of viral replication seen in these two animals (see Tables S3 and S4 in the supplemental material) (30, 50).

DISCUSSION

In summary, NFb12 possesses enhanced affinity for FcγRIIIa and enhanced FcγRIIIa-mediated ADCC *in vitro* relative to the wild-type antibody in a series of assays. However, no enhanced protection against SHIV challenge was achieved by treating rhesus macaques with NFb12 compared to wild-type b12 in this *in vivo* model.

The combination of data from previous studies in a single high-dose challenge model and a repeated low-dose challenge model (21, 22) has provided evidence that a b12 antibody deficient in FcγR binding (LALA) has diminished protective capacity relative to wild-type b12. In the high-dose challenge study, treatment with the LALA variant left four out of nine animals unprotected to develop high primary viremias, and three of the animals succumbed to AIDS. In contrast, only one animal out of nine was left unprotected from the group treated with wild-type b12, and this animal had a lower primary viremia (21). In the repeated low-dose study, the LALA variant reduced the risk of infection 10-fold at each challenge compared to a control antibody, whereas the wild-type b12 reduced the risk 21-fold (22). In addition, the presence of wild-type b12 resulted in a lower viral load in infected animals than the LALA variant.

ADCC has often been regarded as the prime candidate mechanism involved in the above-mentioned studies as well as in others investigating Fc-mediated antibody protection mechanisms against HIV (3, 21, 22, 24, 28, 43). However, most evidence has been indirect, typically based on measuring *in vitro* ADCC activity on serum/plasma samples from rhesus macaques or patients. Interestingly, very few HIV-specific ADCC assays, if any, use primary target/effector cells and replicating virus. To enable a reliable signal, most assays (including our own) use engineered target and effector cell lines highly optimized for killing or use target cells coated with a large amount of HIV gp120 protein or peptides (18, 27, 36, 42). The need for these highly optimized assays and the difficulty in measuring ADCC in HIV-infected primary CD4 cells with primary effector cells engender speculation that ADCC may not be a particularly effective anti-HIV mechanism *in vivo*.

The macaque data presented here certainly militate against the involvement of FcγRIIIa-mediated ADCC as a major HIV protective mechanism. However, our results do carry several caveats worth considering: (i) the results apply to a single antibody (b12) in protection against a single virus ($SHIV_{SF162P3}$), making the study of more antibodies with different specificities (perhaps in combinations) desirable for general conclusions to be unambiguously drawn; (ii) there is a formal possibility that the level of ADCC mediated by wild-type b12 in this *in vivo* model is already maximal and therefore cannot be enhanced *in vivo* by the nonfucosylated variant and that a less potent neutralizing (or nonneutralizing) antibody could be speculated to provide higher sensitivity in detecting an effect of enhanced ADCC; (iii) only FcγRIIIa-mediated effector functions were investigated here, but FcγRI and FcγRIIa could play an important role also in ADCC (11, 46).

Nevertheless, our results do suggest that other Fc receptors and/or other FcγR-mediated activities, such as phagocytosis and viral trapping, may be important in antibody protection against HIV and that these mechanisms should now be investigated.

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