JCI The Journal of Clinical Investigation

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Megan C. Wise, … , Laurent Humeau, David B. Weiner

J Clin Invest. 2019. https://doi.org/10.1172/JCI132779.

Research In-Press Preview AIDS/HIV Immunology

Interventions to prevent HIV-1 infection and alternative tools in HIV cure therapy remain pressing goals. Recently, numerous broadly neutralizing HIV-1 monoclonal antibodies (bNAbs) have been developed which possess the characteristics necessary for potential prophylactic or therapeutic approaches. However, formulation complexities especially for multi-antibody deliveries, long infusion times, and production issues could limit the use of these bNAbs when deployed globally impacting their potential application. Here, we describe an approach utilizing synthetic DNA-encoded monoclonal antibodies (dMAbs) for direct in vivo production of prespecified neutralizing activity. We designed 16 different bNAbs as dMAbs cassettes and studied their activity in small and large animals. Sera from animals administered dMAbs neutralized multiple HIV-1 isolates with similar activity to their parental recombinant MAbs. Delivery of multiple dMAbs to a single animal led to increased neutralization breadth. Two dMAbs, PGDM1400 and PGT121, were advanced into non-human primates for study. High peak circulating levels (between 6-34μg/ml) of these dMAbs were measured and the sera of all animals displayed broad neutralizing activity. The dMAb approach provides an important local delivery platform for the in vivo generation of HIV-1 bNAbs and for other infectious disease antibodies.

In vivo delivery of synthetic DNA-encoded antibodies induces broad HIV-1-neutralizing activity.

Megan C Wise[1#], Ziyang Xu[2,3#], Edgar Tello-Ruiz[2], Charles Beck[4], Aspen Trautz[2], Ami Patel[2], Sarah TC Elliott[2], Neethu Chokkalingam[2], Sophie Kim[2], Melissa G Kerkau[4], Kar Muthumani[2], Jingjing Jiang[1], Paul Fisher[1], Stephany J Ramos[1], Trevor RF Smith[1], Janess Mendoza[1], Kate E Broderick[1], David C Montefiori[5], Guido Ferrari[4], Daniel W Kulp[2], Laurent Humeau[1], David B Weiner[2*]

[1]Inovio Pharmaceuticals, Plymouth Meeting, PA, USA

[2]Vaccine and Immunotherapy Center, The Wistar Institute, Philadelphia, PA, USA

[3]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

[4]Duke Human Vaccine Institute, Duke University School of Medicine, Durham, NC, USA

[5]Department of Surgery, Duke University, Durham, NC, USA

[#]These authors contributed equally

*To whom correspondence should be addressed: David Weiner, Vaccine and Immunotherapy Center, Wistar Institute, 3601 Spruce Street Room 630, Philadelphia PA, 19104; dweiner@wistar.org; 215-898-0381

Conflicts of Interest

M.C.W., J.J, P.F, S.J.R, T.RF.S, J.M., K.E.B., and L.H. are employees of Inovio Pharmaceuticals and as such receive salary and benefits, including ownership of stock and stock options. K.M. receives grants and consulting fees from Inovio related to DNA vaccine development. D.B·W. has received grant funding, participates in industry collaborations, has received speaking honoraria, and has received fees for consulting, including serving on scientific review committees and board series. Remuneration received by D.B·W. includes direct payments, stock or stock options, and in the interest of disclosure he notes potential conflicts associated with his work with Inovio and possibly others. M.C.W. and D.B.W. have a filed patent 206194-001-P1-US.606207.

Abstract

Interventions to prevent HIV-1 infection and alternative tools in HIV cure therapy remain pressing goals. Recently, numerous broadly neutralizing HIV-1 monoclonal antibodies (bNAbs) have been developed which possess the characteristics necessary for potential prophylactic or therapeutic approaches. However, formulation complexities especially for multi-antibody deliveries, long infusion times, and production issues could limit the use of these bNAbs when deployed globally impacting their potential application. Here, we describe an approach utilizing synthetic DNA-encoded monoclonal antibodies (dMAbs) for direct in vivo production of prespecified neutralizing activity. We designed 16 different bNAbs as dMAbs cassettes and studied their activity in small and large animals. Sera from animals administered dMAbs neutralized multiple HIV-1 isolates with similar activity to their parental recombinant MAbs. Delivery of multiple dMAbs to a single animal led to increased neutralization breadth. Two dMAbs, PGDM1400 and PGT121, were advanced into non-human primates for study. High peak circulating levels (between 6-34μg/ml) of these dMAbs were measured and the sera of all animals displayed broad neutralizing activity. The dMAb approach provides an important local delivery platform for the in vivo generation of HIV-1 bNAbs and for other infectious disease antibodies.

Introduction

In just over four decades since its global emergence, the AIDS epidemic has taken millions of lives. While there have been exceptional advances in antiretroviral therapies, there remains a need for preventive treatments and interventions to eliminate HIV-1 infection (1). In recent years, multiple monoclonal antibodies with potent neutralization capacity have been isolated from HIV-1 infected persons (2, 3). A few of these broadly neutralizing antibodies (bNAbs) have demonstrated efficacy in preventing infection after a single dose of intravenous recombinant protein in non-human primates (4). Such observations have generated enthusiasm in the field and progressed HIV-1 bNAbs into the clinic for studies of prevention (NCT02256631, NCT02568215, NCT02716675) as well as for HIV treatment towards cure strategies (5-9). Recently, clinical trials have explored the capability of these antibodies to lower viral loads or prevent rebound after analytical treatment interruption (ATI) (8, 9). Most notably, a study by Mendoza et al. demonstrated that a combination of two bNAbs, 3BNC117 and 10-1074, prevented viral rebound for a median of 21 weeks in a subset of individuals compared to 2.3 weeks in historical controls (6).

The widespread use of passive delivery of recombinant antibodies is impacted due to infusion time, formulations issues, product temperature stability, re-dosing requirements and significant manufacturing costs (10). Viral vector delivery with adeno-associated virus (AAV) has been previously evaluated as a delivery platform for HIV-1 bNAbs, with high-level and long-term expression of the transgene antibody (11-13). However, AAV delivery can be limited in populations by pre-existing neutralizing antibodies to the vector, safety concerns of permanent gene marking of the patient, temperature stability, manufacturing cost, as well as vector seroconversion potentially preventing re-administration ultimately resulting in reduced antibody levels in many subjects (14). Recent clinical results of recombinant AAV-1 delivered PG9 demonstrated limited detection of circulating PG9 in healthy males who were delivered a range of vector doses ($4x10^{12}$ – $1.2x10^{14}$ vector genomes) (15). In this study, we

explored the use of synthetic DNA encoded monoclonal antibodies (dMAb) as a possible alternative, serology-independent, approach to passive transfer and AAV delivery. Upon injection and electroporation of optimized plasmid DNA with transgenes encoding antibody, locally transfected cells become the in vivo bio-factory for antibody production. We have previously demonstrated this dMAb technology was able to induce protective levels of antibody in mice against important infectious disease targets, including influenza A and B viruses, *Pseudomonas aeruginosa*, Zaire Ebolavirus, dengue virus, Zika virus and chikungunya virus (16-21).

Here, we studied the activity of a panel of 16 engineered dMAbs encoding HIV-1 specific monoclonal antibodies that exhibit broad neutralizing activity. Following dMAb administration, we observed rapid expression and sustained blood levels for months in small animals. These in vivo produced dMAbs were functionally active and neutralized to varying degrees the 12 global panel viruses in an envelope-pseudotyped virus neutralization assay (22). To decrease the possibility of viral escape, we next explored administration of multiple (up to four) dMAbs into a single animal and demonstrated expansion of serum neutralizing breadth. Based on in vivo dMAb levels and neutralizing potency, we advanced two dMAbs, PGT121 and PGDM1400, for a pilot non-human primate (NHP) study. In this study, NHP were delivered a single dMAb (PGDM1400) or a combination of the two (PGT121 and PGDM1400). Strong expression was observed in both groups with a range from 6-34.3 μg/ml at peak levels. All animals expressed dMAbs and the serum demonstrated strong tier-2 neutralization breadth. Additionally, the levels of dMAb observed in this study were, on average, more than ten times higher compared to an initial NHP dMAbs study (21). This provides evidence for further development of this platform, which represents an alternative modality for in vivo antibody production against HIV-1 and other biological targets.

Results

Robust expression of optimized HIV-1 dMAbs expressed in vitro and in vivo

dMAbs utilize optimizations that were developed to increase plasmid uptake and expression in context of delivery by adaptive electroporation (23). These improvements resulted in significant enhancement of in vivo expression launched from plasmids. Here we adapted multiple HIV-1 specific bNAbs and tested their expression and in vivo levels. We compared 16 different bNAbs targeting five different regions of the HIV-1 envelope: CD4 binding site (VRC01, N6, 12A21, 3BNC117, IOMA, NIH45-46); high mannose glycan patch (PGT121, PGT128, 10-1074, PGT130); apex (PGT145, PGDM1400, PG9); gp120-gp41 interface (PGT151, 35O22) and gp41 fusion domain (VRC34.01) (2, 24-27). Broadly neutralizing antibodies encoded as dMAbs were selected based on target epitope, neutralization capacity and other characteristics such as length of heavy chain complementarity-determining regions 3 (CDR3) and percent of somatic hypermutation to obtain a range of antibody characteristics (Table S1). The heavy chain length of the CDR3 region ranged from 12 (3BNC117) to 35 (PGDM1400) with an average length across all dMAbs of 22. The percent of amino acid somatic mutations from germline in the heavy chain ranged from 18% (IOMA) to 42% (VRC01) with an average of 28%. Plasmids encoding the heavy chain or the light chain of each of the broadly neutralizing antibodies were RNA and codon optimized, synthesized and cloned into the modified pVax1 backbone. All dMAbs were of the human IgG1 isotype. Expression levels in vitro of all dMAbs were confirmed using transient transfection of HEK 293T cells (**Figure S1**).

Next we proceeded to assess in vivo expression in transiently immunodepleted mice to prevent the development of anti-drug antibodies responses against the human IgG1. Mice were injected with plasmid dMAb constructs followed by intramuscular electroporation (IM-EP) using the CELLECTRA® 3P device. We observed dMAb expression in sera two days post injection with peak levels around Day 21 (**Figure S2**). dMAb was continuously detected in the sera for over 300 days. Peak levels of dMAbs varied from below 1μg/ml to greater than 80μg/ml (**Figure 1A**). The majority of dMAbs exhibited peak levels

between 10-30μg/ml. There was minimal variability among mice receiving a given dMAb, supporting a model where the intrinsic properties of each antibody sequence influence the overall levels observed in vivo. We did not observe any correlation in dMAb levels for different families, heavy chain CDR3 length or percent somatic hypermutation rate of the heavy chain.

Functionality of in vivo produced dMAbs is comparable to recombinant MAb counterparts

To further characterize in vivo produced dMAbs, we investigated their ability to bind to trimer as compared to recombinantly produced monoclonal antibodies. We observed similar strength of binding to trimer as compared to recombinant protein monoclonal antibody for all tested dMAbs (**Figure 1B and Figure S3A**). In agreement with our other dMAb studies, this suggests proper folding of the dMAb in vivo and supports retention of their antigen specificity (16-20).

We next examined dMAb functionality in the context of neutralization using HIV-1 envelope pseudotyped viruses representing the global diversity of HIV-1 glycoprotein (22). We observed strong neutralization titers for all the studied dMAbs (**Figure 1C-D**). Importantly, there was low variability in neutralization titers among mice given a specific dMAb based on each specific group and serum neutralization titers (IC_{50}) were similar to titers reported in the literature (**Figure 1C and Figure S3B**). The neutralization data further confirm dMAbs were assembled, formed and properly folded in vivo and then exhibit similar potency to the recombinant protein monoclonal antibodies.

Modifications improved production of low expressing N6 dMAb

N6 is an extremely potent and broad neutralizing anti-HIV-1 antibody (24), however its in vivo levels were among the three lowest (**Figure 1A**). Based on these previous studies, we observed that single amino acid modification can significantly increase dMAb expression (16). Thus, we sought to increase its expression by designing modifications to both the heavy chain (HC) and light chain (LC) of the original N6 amino acid sequence at the C- and N-terminus of the variable region. These modifications were selected to make the antibody more similar to the human parental germline antibody sequence (**Figure S4A**). Mice

injected with HC unmodified + LC modified (LC_{mod}) or HC modified + LC unmodified (HC_{mod}) dMAb had 3.5-fold increases in levels over the unmodified original N6 (**Figure S4B**). When both modified plasmids were used to assemble modified N6 ($N6_{mod}$), levels increased 9-fold over unmodified and a 2.5-fold over each modified chain. Modifications to the variable regions can change an antibody's ability to bind to its target and impact its functionality. Binding to trimer was similar between serum from mice injected with $N6_{mod}$ dMAb and recombinant unmodified N6 (**Figure S4C**). Furthermore, recombinant antibodies expressing either N6 or $N6_{mod}$ were able to neutralize multiple viruses from the global panel to similar degrees as levels previously reported in the literature (**Figure S4D**)(24). Thus, these modifications appear to be important for increasing overall production in vivo, resulting in increased serum levels of antibody while maintaining the functionality of this antibody. While there was marked improvement in the in vivo production levels of $N6_{mod}$, these levels remain on the lower end and additional rounds of optimization could further improve in vivo levels.

Delivery of multiple dMAbs to provide enhanced coverage of viral mutations

Due to the high error rate of HIV-1 reverse transcription and resultant high antigenic variability, viral immune escape from a single-antibody therapy is likely (7). Additionally, escape mutations to the mAb may already exist in populations as no single mAb targets all circulating HIV-1 strains (7). In order to overcome such issues, we evaluated co-delivery of multiple bNAbs against distinct HIV-1 envelope epitopes in the dMAb delivery platform. We selected two combinations which are currently in clinical trials, 3BNC117 + 10-1074 (5, 6) and PGDM1400 + PGT121 (NCT0320591), as well as PGT121 + PGT145. PGT121 and PGT145 were chosen based on in vivo dMAb levels, target epitope and neutralization profile of the antibodies. Mice were dosed with either a single dMAb construct or with two dMAb constructs in separate distinct muscle sites. In vivo levels of each individual antibody were similar in the combination mice as compared to mice delivered only a single antibody (**Figure 2A**). Individually, bNAb targets between 7 and 11 viruses in the global panel with various gaps in neutralization capacity (**Figure 1C**). By expressing

two dMAbs in a single mouse, we observed an increase in their overall breadth of neutralization, targeting never less than 11 different members of the global panel as compared to each of the individual dMAbs with the PGDM1400 + PGT121 (NCT0320591) combination now providing 100% viral coverage (**Figure 2B**)(28).

We next sought to deliver and express four dMAbs in a single mouse using antibodies PGDM1400, PGT151, VRC01, and PGT121. Such deliveries of multiple antibodies are difficult in other methods. For this study the antibodies were selected based on their neutralization capacity, overall in vivo levels, and ability to target distinct epitopes on the HIV-1 envelope. In these studies, animals were injected with a single dMAb or with all four. As we do not have anti-idiotype antibodies for these antibodies, we measured the total amount of the xenogeneic human antibody expressed in the mice (**Figure 2C**). The total serum hIgG1 dMAb levels in the mice administered with all four dMAb constructs were comparable to the sum of the levels of each dMAb construct administered individually (sum of mice injected the individual dMAbs: 26.01μg/ml vs combination dMAb mice: 25.10μg/ml). Once again, we observed increased neutralization breadth in the sera of mice that received all four dMAb constructs compared to neutralization breadth in the sera of mice that received each individual dMAb construct (**Figure 2D**). By delivering all four dMAb constructs at once, we observed neutralization IC_{50} levels below 0.1μg/ml across the entire global panel.

HIV-1 dMAbs express in NHPs

Based on the promising studies in mice, we next explored dMAb delivery of HIV-1 specific dMAbs in a pilot NHP animal model which is more relevant for translation to humans. Two dMAbs were selected to move into NHPs, PGDM1400 and PGT121, based on high in vivo dMAb levels in mice (**Figure 1**). Two groups of four macaques were dosed with either 6mg of PGDM1400 dMAb plasmid construct (Group 1) or 3mg of PGDM1400 plus 3mg of PGT121 dMAb plasmid construct (Group 2). Expression of human IgG1 (hIgG1) was detected in NHP serum as early as 3 days post-injection and peaked at Day 14 (**Figure 3A,**

Figure S5A and S6A). Total human IgG1 levels at peak were slightly higher for the group receiving PGDM1400 dMAb alone (Group 1) compared to the two dMAbs PGDM1400 and PGT121 (Group 2) (**Figure 3B**). The total hIgG1 detected in the serum from Group 1 ranged between 11.2 and 34.3μg/ml (mean 25.1μg/ml) and for Group 2 between 6.3 and 20.4μg/ml (mean 10.1μg/ml). The levels of human IgG1 dMAb in the sera declined after Day 14 to undetectable levels by Day 35, which is expected in this context where a xenogeneic human IgG was being expressed in an immune-competent NHP host (29-31). Accordingly, the decrease in dMAb levels after Day 14 corresponded with the development of NHP anti-human IgG-dMAb antibodies in the sera (**Figure S5B and S6B**). Using envelope antigen and secondary antibodies specifically recognizing hIgG1 kappa (PGDM1400) versus hIgG1 lambda (PGT121) light chains, we were able to confirm expression of both PGDM1400 and PGT121 dMAbs in Group 2 NHP sera (**Figure 3C, S5C and S6C**).

We proceeded to determine the anti-viral activity of the sera harboring the anti-HIV-1 dMAbs. Pre-bleed sera (Day 0 (D0)) and sera from the peak dMAb level time point (Day 14 (D14)) were tested for neutralization against the global panel tier-2 viruses. NHP sera contained no neutralizing antibodies before dMAb administration (D0) and no non-specific neutralization was detected against mouse leukemia virus (MLV) on D0 and D14 (**Table 1**). Sera collected at peak dMAb levels were able to neutralize 11 out of 12 viruses (Group 1) and 12 out of 12 viruses (Group 2) (**Figure 3D and Table 1**). For several viruses, specifically 243F6, 25710, CE0217, CNE55, and CNE8, titers (IC_{50}) for both groups were less than 0.1μg/ml. These pseudotype neutralization titers, originally performed at The Wistar Institute, were then retested and confirmed at Duke University. Similar Group 1 and Group 2 NHP neutralization titers (IC_{50}) were obtained across the 9 HIV-1 pseudotype viruses re-evaluated (**Figure S7A**). Delivery of a second dMAb in Group 2 modestly improved neutralization for some isolates and added neutralization coverage for two additional viruses, 398F1 and TRO.11. We further explored the anti-viral activity by exploring the antibody-dependent cell-mediated cytotoxicity (ADCC) against a subtype C HIV-1 infectious molecular

clone (IMC) DU151 infected cells, chosen based on the sensitivity to both mAbs. Though ADCC activity of the serum was usually only detected at concentration >4μg/mL, it was comparable to the recombinant protein monoclonal antibody activity (**Figure 3E**). No ADCC activity was observed for the negative control recombinant Palivizumab antibody (**Figure S7B**). In summary, the NHP data support that HIV-1 dMAbs expressed at high levels, bind to envelope trimers, neutralize numerous tier-2 viruses and have effector functions and can complement each other in vivo.

Discussion

Recently, the use of protein monoclonal antibodies has become a first-line treatment for numerous cancers, and similarly plays a major role in autoimmune disease therapies (32). In general though, the adoption of MAb for infectious disease is very exciting but to date there have been limited approvals (32). Due to their exceptional breadth and potency, clinical trials are in progress to explore the ability of broadly neutralizing antibodies (bNAbs) against HIV-1 to both prevent and treat infection (7). Additional strategies for delivery are likely important especially for providing these strategy in the developing world (33). In this manuscript, we describe a recently developed DNA-encoded monoclonal antibody (dMAb) platform for delivering bNAbs and provide the first proof of concept for this delivery targeting HIV-1.

Through iterative studies, we describe dMAb delivery resulting in expression of multiple HIV-1 specific antibodies up to 80µg/ml in mice. To date, in vitro expression of bNAbs from transfected cell lines does not correlate or predict in vivo levels when delivered via the dMAb platform (16). Interestingly, similar variations in dMAb levels across multiple different antibodies were observed with AAV-mediated gene delivery (12, 29, 34). Even within the same class of antibodies which share the germline VH gene (IGHV1-2) usage, dMAb levels vary significantly between 1.3µg/ml (N6) and 52.2µg/ml (IOMA) (**Figure 1A**). Furthermore, we did not observe any correlation of in vivo levels and heavy chain CDR3 length, light chain usage or rate of somatic hypermutation. We demonstrate that modifications to the beginning and end of N6 variable regions of the heavy and light chains improved in vivo levels while maintaining the antibody's activity (**Figure S4**). As we acquire more data on antibody sequences and dMAb expression, a better understanding of in vivo sequence liabilities will be obtained. As HIV-1 bNAbs are highly somatically mutated, and many of these mutations are required for maintaining the functionality of the antibody, balancing mutations made for increasing dMAb levels will need to be weighted with impacts on functionality. DNA encoded monoclonal antibodies are an important tool for these studies since DNA can

be easily modified to encode different amino acids and the effects on both in vivo expression as well as functionality can be quickly and cost-effectively explored. Furthermore, the monoclonal antibody sequence rules which dictate the in vivo expression of dMAbs delivered to muscle tissue could translate to other platforms such as AAV where muscle expression is also being tested.

We demonstrated the ability to encode two HIV-1 specific bNabs in NHPs using the dMAb technology. We observed dMAb expression in NHP sera within three days of dMAb administration and levels that peaks around Day 14. We believe these peak levels (ranging from 6 to 34μg/ml) would be protective against multiple SHIV strains upon challenge based upon prior studies which utilized recombinant monoclonal antibody protein (4, 28, 35-37). Specifically, Julg et al. demonstrated that NHPs delivered passive infusions of 2 and 0.4mg/kg of PGDM1400 one day before challenge with SHIV-325c were protected from infection (28). The average level of PGDM1400 in the serum at time of challenge was 6.9 and 2.5 μg/ml for 2 and 0.4mg/kg infusions respectively. There was breakthrough infection in the group delivered 0.08mg/kg which corresponded to a serum level of 0.22 μg/ml at time of challenge. Additionally, PGT121 has demonstrated protection against SHIV-SF162P3 and SHIV-AD8EO at levels of 15 and 22 μg/ml respectively with partial infection at serum levels of 1.8 μg/ml (36, 37). In the context of treatment, NHPs chronically infected with SHIV-SF162P3 and delivered a single infusion of 10mg/kg of PGT121 were able to control viral loads to undetectable levels (38). Rebound occurred in 3 out of 4 animals once mAb serum concentrations reached undetectable (<1μg/ml) with one animal having long-term virologic control. While we are unaware of a prior study for the use of PGDM1400 in pre-clinical treatment setting, the peak levels of this antibody in the NHPs might be relevant in therapeutic settings especially considering that the mean concentration of 3BNC117 and 10-1074 were between 1.9 and 14.8 μg/ml at the time of viral rebound in the study by Mendoza et al (6). However, additional testing of the ability of dMAbs, that are developed as species matched antibodies, to impact challenge outcomes or control of infection will be informative.

There was a decline in human dMAb levels the NHP study observed after Day 14 corresponds with the development of anti-drug antibodies (ADA) due to the expression of the xenogeneic protein. The development of ADA against a cross-species human IgG has also been observed in NHPs following AAV , recombinant protein (30), as well as RNA (39) and adenovirus delivery of antibodies in mice (40). In studies of AAV delivery of HIV-1/SIV bNAbs, induction of ADA was closely associated with the distance of the variable regions from germline (31). Furthermore, AAV delivery of fully rhesus monoclonal antibodies against SIV led to a lower incidence of ADA responses in rhesus macaques (34). In mice, we eliminated host immune responses to human IgG1 dMAb via transient immunosuppression. Additionally, we have observed that using a species matched fully murine dMAb in mice can avoided significant ADA development and allowed for dMAb detection in the serum of several months (41). However, we have not yet developed fully simian dMAbs and future studies will test this principle. Ultimately, human studies will be particularly informative. In this regard, recent monoclonal antibody delivery clinical trials include an AAV vector encoding HIV-1 bNAb VRC07 (NCT03374202), the first dMAb construct for expression of antibody against Zika virus (NCT03831503), and an mRNA platform for delivery of anti-chikungunya virus antibody (NCT03829384) will be particularly valuable to provide additional information on this important question.

Depending on the application of the monoclonal antibody, different effector functions and modifications in the antibody Fc domain may be required. Monoclonal antibodies used for HIV-1 prevention and treatment will likely benefit from longer in vivo half-lives. Amino acid mutations to the Fc, including YTE and LS, yield prolonged recombinant in vivo antibody half-lives in both preclinical NHP studies and in the clinic (42). We have previously demonstrated the feasibility of dMAbs to encode Fc modifications such as the LALA mutation to prevent antibody dependent enhancement (ADE) in dengue virus infection (20). Furthermore, modifications to the Fc region of the antibody can increase ADCC activity which is important in HIV-1 protection and control of infection (43). Inclusion of these modifications to

increase activity of dMAb against infected cells is imperative for therapeutic cure approaches. Exploration of half-life extension and Fc activity modification for HIV-1 dMAbs is ongoing.

Our data describe a new important technology for in vivo HIV-1 antibody delivery. We demonstrated that dMAb constructs can be developed to encode multiple HIV-1 specific IgG1 and that the in vivo expressed dMAbs retain their functional activity in both small and large animals. The observation that in vivo produced dMAbs had similar HIV-1 envelope trimer binding and neutralization capacity as recombinantly produced counterparts highlights the effective folding of the dMAb antibody in this system. Furthermore, the ability to deliver multiple antibodies, in this case four at one time, to limit viral escape and resistance may be important for HIV-1 prevention and treatment strategies. To our knowledge, this is the most comprehensive screening of multiple HIV-1 antibodies delivered by the same platform, demonstrating how inherent antibody characteristics influence in vivo production. Additionally, through numerous optimization efforts aiming at both the DNA synthetic design and the delivery technology, we were able to consistently reach greater than 5μg/ml of antibody in NHPs, with some NHPs reaching serum levels of more than 30μg/ml. These levels represent a significant improvement compared to the original NHP dMAb studies , which achieved on average a tenth the in vivo production observed here (21). These studies demonstrate the possibility of dMAbs as an approach to delivery of monoclonal antibody specificities in a simple to produce, temperature stable, and rapid delivery format. Further study of the dMAb platform for anti-HIV strategies appears important.

Methods

DNA design and plasmid synthesis

Protein sequences for HIV-1 antibodies were obtained from NCBI GenBank (**Table S2**). The DNA sequences were RNA and codon optimized as previously described (16-18, 20). Leader sequences were added to the beginning of the heavy and light chain of the antibody. The heavy and light chain were synthesized and cloned (Genscript) into a modified mammalian pVAX1 expression plasmid (ThermoFisher). Plasmids encoding the 12 global panel HIV envelope gp160 for the TZM-bl neutralization assay, and a plasmid encoding the Murine Leukemia Virus (MLV) envelope as an antibody-non-specific pseudotype control, were obtained from NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

Cell lines, transfection and recombinant antibody purifications

HEK 293T cells (ATCC) and TZM-bl cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) were maintained in Dulbecco's Modified Eagle medium (DMEM) (ThermoFisher) supplemented with 10% of heat inactivated fetal bovine serum (Atlas Biologicals). Expi293F cells (ThermoFisher) were maintained in Expi293 expression medium (ThermoFisher). All cell lines were mycoplasma negative. HEK293T cells were transfected with HIV-1 bNAb-encoding dMAb plasmids using GeneJammer (Agilent) transfection reagent following the manufacturer's protocol. Two days after transfection, media and cells were harvested and lysed with cell lysis buffer (Cell Signaling Technology) modified with cOmplete^{TM} EDTA-free protease inhibitor cocktail (Roche). To produce recombinant HIV monoclonal antibodies for assay and controls, Expi293F cells were transfected following manufacturer's protocol for Expifectamine (ThermoFisher). Transfection enhancers were added 18 hours after transfection and supernatants were harvested 6 days after transfection. Protein G agarose (ThermoFisher) was then used following manufacturer's protocol to purify the IgG. Recombinant antibody purity was confirmed with Coomassie staining of SDS-PAGE gels and quantified using the quantification ELISA described below.

Trimer production

Expi293F cells were transfected with plasmid expressing the HIV-1 gp160 Env trimer BG505_MD39_His construct. Cell supernatants containing trimer were clarified by centrifuging (4000xg, 25mins) and filtering (0.2um Nalgene Rapid-Flow Filter). Trimers were then purified from supernatants by nickel affinity chromatography on a HIS-TRAP HP column (GE Healthcare). The trimers were then purified over a size-exclusion chromatography column (GE S200 Increase) in PBS. The molecular weight and homogeneity of the trimers were confirmed by protein conjugated analysis from ASTRA software (Wyatt Technology) with data collected from a size-exclusion chromatography-multi-angle light scattering (SEC-MALS) experiment run in PBS using a GE S6 Increase column, followed by DAWN HELEOS II and Optilab T-rEX detectors. The trimers were aliquoted at 1mg/ml and flash frozen in thin-walled PCR tubes prior to use.

ELISA

dMAb quantification ELISA- mouse

DNA encoded monoclonal antibody levels were quantified as previously described (16). Briefly, high binding polystyrene 96 well plates (ThermoFisher) were coated with unconjugated purified goat anti-human IgG-Fc (1µg/ml) overnight in PBS. After blocking with 10% newborn calf serum (NCS) plates were washed with PBS containing 0.05% Tween-20. Mouse serum and standards were serially diluted and incubated for one hour at room temperature. Purified human IgG-Kappa (P80-111, Bethyl Laboratories) and human IgG-Lambda (P80-116, Bethyl Laboratories) were used as standards. After washing, plates were incubated with 1:20,000 dilution of either goat anti-human kappa (A80-115P, Bethyl Laboratories) or goat anti-human lambda (A80-116P, Bethyl Laboratories) light chain secondary antibodies conjugated to horseradish peroxidase (HRP) for one hour at room temperature. For quantification of total antibody levels in the mice dosed with four dMAb constructs, a secondary goat anti-human IgG H+L conjugated to horseradish peroxidase (A80-119P, Bethyl Laboratories) was used. After washing, plates were developed with o-phenylenediamine dihydrochloride (OPD) substrate (SIGMAFAST OPD, Sigma Aldrich). Plates were stopped with 2N H_2SO_4. Plates were read on a BioTek Synergy 2 plate reader (BioTek) at 450nm and 570nm

wavelengths. Quantification of serum dMAb levels were determined by interpolating the unknown OD values to the standard curve.

dMAb quantification ELISA- Non-human primate

Quantification for human IgG dMAb in NHP sera was determined using the human therapeutic IgG1 ELISA kit from Cayman Chemicals following the manufacturer's protocol. Serum was serially diluted to obtain two OD values within the linear range of the standard curve. Quantification measurements were repeated twice for Days 7-21.

dMAb binding to trimer ELISA

Binding curves of HIV serum-expressed dMAbs compared to recombinant proteins were obtained by coating 96 well half-area high binding polystyrene plates (ThermoFisher) with 1µg/ml of rabbit anti-His antibody (25B6E11, Genscript) in PBS at 4^{o}C. Plates were blocked with 5% skim milk in PBS with 1% newborn calf serum (NCS) (Atlas Biologicals) and 0.2% Tween-20 for one hour at room temperature. His-BG505 MD39 trimer protein was then added at 1µg/ml in PBS with 1% NBS and 0.2% Tween-20 for two hours at room temperature. Serum was normalized based on the quantification concentration and serially diluted 2-fold from there. A similar amount of purified recombinant monoclonal antibody was added to match the serum concentration. Plates were incubated for one hour at 37^{o}C. After washing, a 1:20,000 dilution of secondary goat anti-human kappa (A80-115P, Bethyl Laboratories) or goat anti-human lambda (A80-116P, Bethyl Laboratories) light chain secondary antibodies conjugated to horseradish peroxidase were added and incubated for one hour at room temperature. Plates were then developed with 1-step ultra-3,3',5,5'- tetramethylbenzidine (TMB) substrate (ThermoFisher) and read on a BioTek Synergy 2 plate reader (BioTek) at 450nm and 570nm wavelengths.

Anti-antibody detection ELISA

To determine the development of anti-drug antibody development, 96 well half-area high binding polystyrene plates (ThermoFisher) were coated with 1µg/ml of purified PGT121 or PGDM1400 (produced

in-house) overnight in PBS at 4°C. Plates were then blocked with 5% skim milk in PBS with 1% newborn calf serum (NCS) (Atlas Biologicals) and 0.2% Tween for one hour at room temperature. NHP sera was diluted 1:100 and added to plates for each time point and incubated for one hour at 37°C. After washing, a 1:20,000 dilution of secondary goat anti-NHP H+L min human secondary antibody conjugated to HRP (A140-202P, Bethyl Laboratories) was then added and incubated for 1hr at room temperature. Plates were then developed with 1-step ultra-TMB substrate (ThermoFisher) and read on BioTek Synergy 2 plate reader (BioTek) at 450nm and 570nm wavelengths.

Western blot

Western blots of transfected cell media were performed using 4-12% Bis-Tris gels run with 2-(N-morpholino) ethanesulfonic acid (MES) buffer (ThermoFisher). The gel was then transferred to a PVDF membrane (Millipore) and blocked with blocking buffer (LI-COR Bioscience) for one hour at room temperature. Membranes were then probed with IRDye-680RD anti-human secondary antibody (925-68078, LI-COR Bioscience) for one hour at room temperature. After washing, the membrane was scanned using the Odyssey CLx (LI-COR Bioscience).

Animals – mice

Groups of five, six to eight week old female BALB/c mice (Charles River) were transiently depleted of T cells as previously described (16) at the same time as dMAb injection to attenuate mouse anti-human IgG anti-antibody immune responses. For experiments where a single dMAb was delivered, mice were injected with 100µg (25µg of heavy chain and 25µg of light chain per site, 2 sites total) dMAb plasmids formulated with hyaluronidase (200U/L, Sigma Aldrich) and injected into the tibialis anterior muscles followed by intramuscular electroporation (IM-EP) using the CELLECTRA® 3P device (Inovio Pharmaceuticals). For experiments with two dMAb delivery, mice were injected with 100µg (25µg of heavy chain and 25µg of light chain per site, 2 sites total) of each dMAb plasmids formulated with hyaluronidase and injected into the tibialis anterior and quadriceps muscles followed by intramuscular

electroporation (IM-EP). For experiments with four dMAb delivery, single dMAb control mice were injected with 50µg (25µg of heavy chain and 25µg of light chain per site, 1 site total), formulated and injected into the tibialis anterior. Mice receiving four dMAb plasmids were injected with 50µg (25µg of heavy chain and 25µg of light chain per site, 1 sites total) of each dMAb plasmid, formulated and injected into the tibialis anterior and quadriceps muscles followed by IM-EP. Mice were serially bled to obtain serum for analysis.

Animals- non-human primates

Two groups of cynomolgus macaques (N=4) (Primgen) were treated with plasmid dMAb constructs encoding either PGDM1400 or PGDM1400 and PGT121, with animals receiving a total of 6mg plasmid DNA. DNA was co-formulated with hyaluronidase (Hylenex – 135 U/ml). Multi-depth IM injection was performed followed by electroporation using the Elgen1000 Twinjector (Inovio Pharmaceuticals). Macaques were serially bled to obtain serum over 60 days.

***Ex vivo* neutralization assay**

Production of HIV envelope pseudotyped viruses and TZM-bl neutralization assay were performed as previously described (44). Pseudotyped viruses were produced using HEK293T cells transfected with 4µg of HIV Env plasmid and 8µg of plasmid encoding the HIV backbone lacking Env (pSG3ΔEnv – NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) using GeneJammer (Agilent). Forty-eight hours post transfection, media was collected, filtered, and aliquoted and stored at -80^{o}C. Viruses titers were determined to yield 150,000 RLU after 48h of infection. Mouse serum was heat inactivated for 15 minutes at 56^{o}C and NHP serum was inactivate for 30 minutes. Serum concentration was determined for 50% virus inhibition dilution (ID_{50}) and calculated using total human IgG quantification levels to determine the IC_{50}.

Infectious molecular clones (IMC)

The HIV-1 reporter virus used were replication-competent IMC designed to encode the *env* genes of DU151 (subtype C; GeneBank No. DQ411851) in *cis* within a Nef deficient isogenic backbone that

expresses the *Renilla* luciferase reporter gene. All the IMCs were built using the original NL-LucR.T2A-ENV.ecto backbone as previously described (45). Reporter virus stocks were generated by transfection of 293T cells with proviral IMC plasmid DNA, and virus titer was determined on TZM-bl cells for quality control.

Infection of CEM.NKR$_{CCR5}$ cell line with HIV-1 IMCs

CEM.NKR$_{CCR5}$ (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) cells were infected with HIV-1 IMCs as previously described (46). Briefly, IMCs were titrated in order to achieve maximum expression within 48-72 hours post-infection as determined by detection of Luciferase activity and intracellular p24 expression. IMC infections were performed by incubation of the optimal dilution of virus with CEM.NKR$_{CCR5}$ cells for 0.5 hour at 37^{o}C and 5% CO_2 in presence of DEAE-dextran (7.5 μg/ml). The cells were subsequently resuspended at 0.5x10^{6}/ml and cultured for 48-72 hours in complete medium containing 7.5μg/ml DEAE-dextran. For each ADCC assay, the frequency infected target cells were monitored by intracellular p24 staining. Assays performed using infected target cells were considered reliable if cell viability was ≥60% and the percentage of viable p24^{+} target cells on assay day was ≥20%.

Luciferase ADCC Assay

ADCC activity was determined by a luciferase (Luc)-based assay as previously described (47). Briefly, CEM.NKR$_{CCR5}$ cells were used as targets after infection with the HIV-1 IMCs. PBMC obtained from a HIV-seronegative donor with the heterozygous 158F/V and 131H/R genotypes for FcγR3A and FcγR2A, respectively, were used as a source of effector cells, and were used at an effector to target ratio of 30:1. Sera from NHPs inoculated with dMAb plasmids for PDGM1400 and PGT121 were initially diluted to reach an initial concentration ranging from 4 to 6μg/ml of dMAb and tested across a range of concentrations using 5-fold serial dilutions. The reference mAbs were tested across a range of concentrations using 5-fold serial dilutions starting at 50 μg/ml. The effector cells, target cells, and Ab dilutions were plated in opaque 96-well half area plates and were incubated for 6 hours at 37^{o}C in 5% CO_2. The final read-out was

the luminescence intensity (relative light units, RLU) generated by the presence of residual intact target cells that have not been lysed by the effector population in the presence of ADCC-mediating mAb (ViviRen substrate, Promega). The percent of specific killing was calculated using the formula: percent specific killing = [(number of RLU of target and effector well − number of RLU of test well)/number of RLU of target and effector well] ×100. In this analysis, the RLU of the target plus effector wells represents spontaneous lysis in absence of any source of Ab. The ADCC detectable in NHP serum is reported after subtracting the activity observed in the serum before dMAb injection (baseline subtracted activity). Results are considered positive if percent specific killing is greater than 15%. The RSV-specific mAb Palivizumab was used as a negative control and a combination of C1/C2 A32, CD4bs CH44, glycosylation site 2G12, and gp41 7B2 mAbs (mAb mix) was used as positive control.

Statistics

All statistics and calculations were performed using GraphPad Prism 7.0. dMAb levels in the serum were determined using standard curve non-linear regression model and interpolating values. ID_{50} values were computed with a non-linear regression model of percentage neutralization vs log reciprocal serum dilution. IC_{50} values were determined using the total human IgG quantification in the serum and the ID_{50} titers. Linear regression analysis of various antibody characteristic to expression levels in vivo were also performed using GraphPad. Two-tailed student t-test or modified ANOVA was used to determine statistical significance. A p-value less than 0.05 was considered significant.

Study approval

All mouse experiments were carried out in accordance with animal protocol 112776 approved by the Wistar Institute Institutional Animal Care and Use Committee (IACUC). For the NHP expression study, cynomolgus macaques were house at Bioqual according to the standards of the American Association for Accreditation of Laboratory Animal Care, and all animal protocols were IACUC approved.

Author Contributions

M.C.W, Z.X, D.W.K, L.H and D.B.W were involved in study concept and design. M.C.W, Z.X, E.T-R, C.B, A.T, A.P, S.TC.E, S.K, M.K, J.J, P.F, S.J.R, T.RF.S, J.M D.C.M, and G.F, were involved in the acquisition, the analysis and the interpretation of data. N.C., K.M., and D.W.K. contributed crucial reagents. M.C.W, Z.X and D.B.W wrote the paper. All authors were involved in critically revising the manuscript. Authorship order for co-first authors was determined based on alphabetical order.

Acknowledgements

We would like to thank the Animal Facility staff at the Wistar Institute for providing house and care to the animals. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Panel of Global HIV-1 Env Clones from Dr. David Montefiori; SV-A-MLV-env from Dr. Nathaniel Landau and Dr. Dan Littman; HIV-1 SG3 ΔEnv non-infectious molecular clone and TZM-bl from Drs. John C. Kappes and Xiaoyun Wu; and CEM.NKR$_{CCR5}$ cells from Dr. Alexandra Trkola.

Funding Sources

This work was supported by an NIH IPCAVD Grant U19 Al109646-04, WW Smith Charitable Trust 6743101374, and Martin Delaney Collaboration HIV Cure Research Grant 2528109374 awarded to D.B.W.

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Figure legends

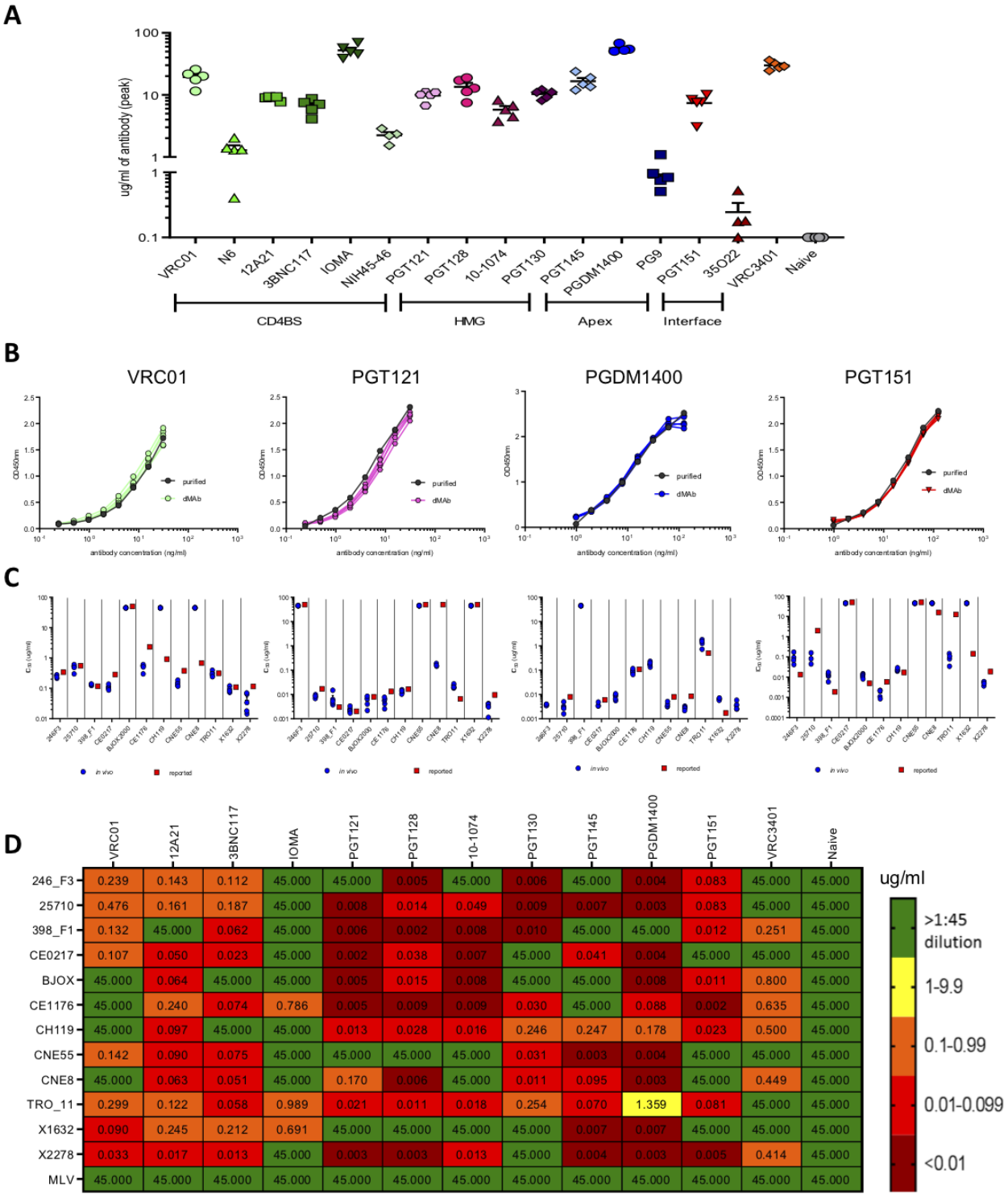
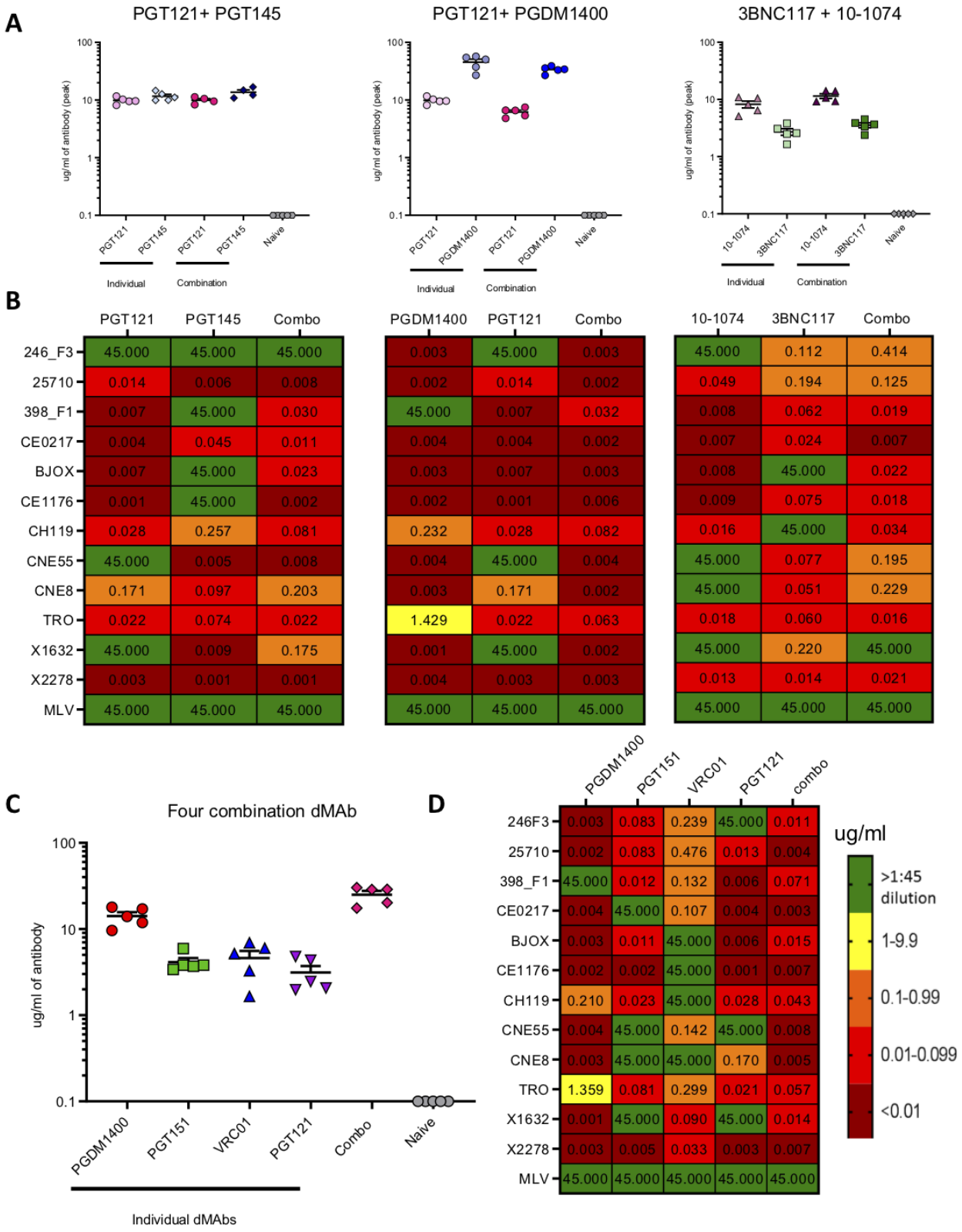
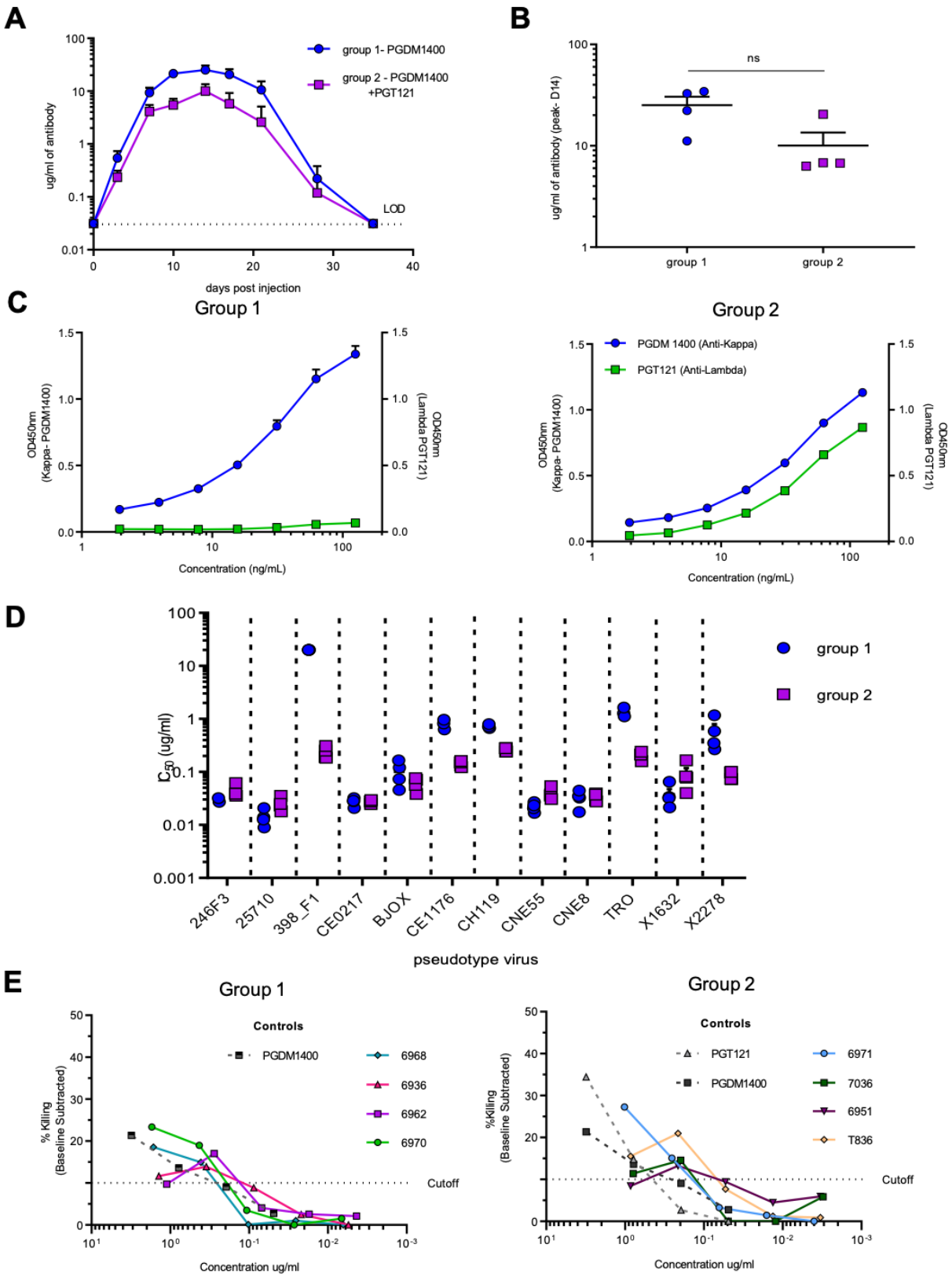


Figure 1: *In vivo expression of dMAb-encoded HIV-1 bNabs in mice.* (**A**) Peak dMAb expression levels (Day 14) of HIV-1 broadly neutralizing antibodies in the sera of transiently immunodepleted mice. Groups of

mice (n=5) were administered dMAb constructs expressing one of sixteen different bNAbs. (**B**) Binding curves for four dMAbs against HIV-1 trimer BG505_MD39. Serum dMAb levels were normalized for expression (colored lines, n=5 mice) and compared to the similar purified recombinant protein (black line) over various concentrations. (**C**) Individual mouse IC_{50} (n=5) for four dMAbs across the 12 viruses global panel (blue circles) vs values reported in the literature (red squares). Literature values gathered from Los Alamos CatNaber. (**D**) Mean (n=5) IC_{50} pseudotype neutralization of Day 14 mouse sera against the 12 viruses of the global panel, and MLV control. Value of 45 corresponds to no neutralization at a 1:45 dilution the lowest dilution tested of the mouse serum. All other values are in µg/ml. Horizontal bars indicate mean; error bars represent standard error of the mean. Expression levels are representative of two experimental replicates, binding and neutralization testing performed once.


A
PGT121+ PGT145
PGT121+ PGDM1400
3BNC117 + 10-1074
ug/ml of antibody (peak)
PGT121
PGT145
PGDM1400
10-1074
3BNC117
Naive
Individual
Combination
B
PGT121
PGT145
Combo
PGDM1400
10-1074
3BNC117
246_F3
25710
398_F1
CE0217
BJOX
CE1176
CH119
CNE55
CNE8
TRO
X1632
X2278
MLV
C
Four combination dMAb
ug/ml of antibody
PGDM1400
PGT151
VRC01
PGT121
Combo
Naive
Individual dMAbs
D
PGDM1400
PGT151
VRC01
PGT121
combo
ug/ml
>1:45 dilution
1-9.9
0.1-0.99
0.01-0.099
<0.01

643 **Figure 2:** *Delivery of multiple dMAb constructs in a single mouse maintains individual dMAb expression*
644 *levels and increases serum neutralization breadth*. (**A**) Groups of mice (n=5) were administered a single
645 dMAb (PG121, PGT145, PGDM1400, 3BNC117 or 10-1074), or a combination of two dMAbs (PGT121 +
646 PGT145, PGDM1400 + PGT121, 3BNC117+10-1074). Peak serum expression levels of human IgG were
647 quantified by ELISA. (**B**) Mean (n=5) IC_{50} pseudotype neutralization against the 12 viruses of the global
648 panel, and MLV control, of sera collected at Day 14 from mice administered a single or two dMAbs. Value
649 of 45 corresponds to no neutralization at a 1:45 dilution the lowest dilution tested of the mouse serum.
650 All other values are in μg/ml. (**C**) Total human IgG serum expression levels following administration of
651 individual dMAbs (PGDM1400, PGT151, VRC01, and PGT121) and co-administration of all four dMAbs
652 (combo) in mice (n=5). (**D**) Mean (n=5) IC_{50} pseudotype neutralization against the 12 viruses of the global
653 panel, and MLV, for sera collected from mice administered individual dMAbs and combination of the four
654 dMAbs. Horizontal bars indicate mean, error bars represent standard error of the mean. Expression levels
655 are representative of two experimental replicates, binding and neutralization testing performed once.
656


A
group 1- PGDM1400
group 2 - PGDM1400 +PGT121
ug/ml of antibody
LOD
days post injection
B
ns
ug/ml of antibody (peak- D14)
group 1
group 2
C
Group 1
Group 2
PGDM 1400 (Anti-Kappa)
PGT121 (Anti-Lambda)
OD450nm (Kappa- PGDM1400)
OD450nm (Lambda PGT121)
Concentration (ng/mL)
D
group 1
group 2
IC50 (ug/ml)
246F3
25710
398_F1
CE0217
BJOX
CE1176
CH119
CNE55
CNE8
TRO
X1632
X2278
pseudotype virus
E
Group 1
Group 2
Controls
PGDM1400
PGT121
6968
6936
6962
6970
6971
7036
6951
T836
% Killing (Baseline Subtracted)
%Killing (Baseline Subtracted)
Cutoff
Concentration ug/ml

657

Figure 3: *PGDM1400 and PGT121 express as dMAbs in NHPs.* Immune competent macaques were injected with human IgG1 (hIgG1) dMAb constructs (Day 0) and serially bled. Group 1 animals (n=4) received 6mg of PGDM1400-encoding plasmid DNA; Group 2 animals (n=4) received 3mg of PGDM1400 and 3mg of PGT121 plasmid DNA. (**A**) Quantification of hIgG1 in the sera of Group 1 and Group 2 NHPs over time. (**B**) Peak expression levels of total hIgG1 for each group at Day 14. (**C**) Serum binding curves against HIV-1 Env trimer, BG505_MD39, using different secondary antibodies to establish the binding of PGDM1400 (human IgG1 kappa light chain, blue), and PGT121 (human IgG1 lambda light chain, green). (**D**) Neutralization IC_{50} of serum across the 12-virus global pseudotype panel using serum from peak dMAb expression (Day 14). (**E**) Baseline subtracted ADCC killing activity of serum for infectious molecular clone DU151 compared to recombinant monoclonal antibodies PGDM1400 and PGT121. Horizontal bars indicate mean; error bars represent standard error of the mean. Expression levels and neutralization titers representative of two replications, all other test performed once. Two-tailed student t-test performed to determine significant difference in levels of expression between group 1 vs group 2. $P < 0.05$ was considered significant.

		246F3		25710		398F1		CE0217		BJOX		CE1176		CH119		CNE55		CNE8		TRO.11		X1632		X2278		MLV	
	NHP ID	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*
Group 1	6970	<20	1262	<20	3834	<20	<20	<20	1646	<20	752	<20	54	<20	51	<20	2041	<20	1049	<20	27	<20	1022	<20	128	<20	<20
	6962	<20	808	<20	1597	<20	<20	<20	795	<20	185	<20	27	<20	31	<20	826	<20	638	<20	20	<20	690	<20	38	<20	<20
	6963	<20	349	<20	880	<20	<20	<20	348	<20	153	<20	<20	<20	<20	<20	539	<20	251	<20	<20	<20	521	<20	32	<20	<20
	6968	<20	994	<20	1571	<20	<20	<20	1149	<20	199	<20	34	<20	41	<20	1403	<20	1877	<20	20	<20	501	<20	28	<20	<20
	average	<20	853.3	<20	1970.5	<20	<20	<20	984.5	<20	322.3	<20	28.8	<20	30.8	<20	1202.3	<20	953.8	<20	20.0	<20	683.5	<20	56.5	<20	<20

		246F3		25710		398F1		CE0217		BJOX		CE1176		CH119		CNE55		CNE8		TRO.11		X1632		X2278		MLV	
	NHP ID	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*	*D0*	*D14*
Group 2	6971	<20	333	<20	581	<20	107	<20	824	<20	286	<20	154	<20	84	<20	568	<20	520	<20	128	<20	123	<20	209	<20	<20
	7036	<20	176	<20	268	<20	34	<20	255	<20	164	<20	51	<20	26	<20	167	<20	216	<20	38	<20	157	<20	86	<20	<20
	6951	<20	175	<20	379	<20	27	<20	245	<20	119	<20	45	<20	24	<20	127	<20	244	<20	32	<20	87	<20	92	<20	<20
	T863	<20	186	<20	270	<20	22	<20	230	<20	89	<20	42	<20	24	<20	218	<20	181	<20	28	<20	82	<20	67	<20	<20
	average	<20	217.5	<20	374.5	<20	60.5	<20	388.5	<20	164.5	<20	73.0	<20	37.0	<20	270.0	<20	290.3	<20	56.5	<20	112.3	<20	113.5	<20	<20

Table 1: *Neutralization ID_{50} of dMAb treated NHP serum across the global panel.* ID_{50} neutralization titers for heat inactivated serum from Day 0 and Day 14 for NHP administered dMAb PGDM1400 (group 1) or PGDM1400 and PGT121 (group 2) across the global panel of pseudotype HIV-1 envelopes and non-specific MLV control.