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EFFECTIVE, LOW TITER, ANTIBODY PROTECTION AGAINST LOW-DOSE REPEATED MUCOSAL SHIV CHALLENGE IN MACAQUES

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Abstract

Neutralizing antibodies are thought crucial to HIV vaccine protection but a major hurdle is the high antibody concentrations likely required as suggested by studies in animal models[1]. However, these studies typically apply a large virus inoculum to ensure infection in control animals in single challenge experiments. In contrast, most human infection via sexual encounter probably involves repeated exposures to much lower doses of virus[2–4]. Therefore, animal studies may have overestimated protective antibody levels in humans. To investigate the impact of virus challenge dose on antibody protection, we repeatedly exposed macaques intravaginally to low doses of a CCR5 coreceptor-using SHIV (an HIV/SIV chimera) in the presence of antibody at plasma concentrations leading to relatively modest neutralization titers of the order of 1:5 IC_{90} values in a PBMC assay. An effector function deficient variant of the neutralizing antibody was also included. The results show that a significantly greater number of challenges are required to infect animals treated with neutralizing antibody than control antibody-treated animals, and the notion that effector function may contribute to antibody protection is supported. Overall, the results imply that lower levels of antibody than considered hereto may provide benefit in the context of typical human exposure to HIV-1.

Much of what we know about antibody protection against HIV comes from studies using passively administered broadly neutralizing human monoclonal antibodies or monospecific neutralizing polyclonal antibodies in animal challenge models[5–11], including intravenous (*i.v.*), vaginal and rectal challenge in macaques. The hallmark of most of these studies is that

AUTHOR CONTRIBUTIONS

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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protection, in the form of sterilizing immunity, is achieved at relatively high serum neutralization titers corresponding to high antibody concentrations. The most quantitative of these studies suggest that sterilizing immunity requires serum antibody concentrations at least two orders of magnitude greater than *in vitro* neutralizing concentrations[10,11]. However, this estimate is quite approximate and dependent upon, among other parameters, the neutralization assay used. Even so, the data have convinced many researchers that achieving sterilizing immunity via antibodies alone is extremely challenging and a more realistic goal for vaccine-induced antibodies has been viewed as blunting infection and relying on vaccine-induced cellular immunity to clear, or, failing that, control infection. However, as noted above, a limitation of macaque protection studies is the use of high viral challenge doses to ensure all control animals become infected with a single challenge. Yet, it is well established that the average probabilities for heterosexual transmission in human exposures are low and dependent upon the viral burden in the donor and susceptibility factors associated with the donor and recipient such as the presence of sexually transmitted diseases (STDs). Transmission frequencies on the order of 1:1,000 per coital act have been reported in chronic infection of the donor[2–4,12], increasing by about an order of magnitude in acute infection[2,3,12,13]. The amount of virus, albeit estimated by quantitative PCR rather than infectivity, contained in a typical macaque challenge is much higher than would be found, for example, in the semen ejaculate of an acutely infected man[12–15]. Indeed, viral inoculums typically average 5×10^5 copies per ejaculate, with a reported maximum of about 2×10^7 copies[12], whereas we found that a high-dose 300 $TCID_{50}$ (50% tissue culture infectious doses) inoculum of $SHIV_{SF162P3}$ contains about 10^8 viral copies.

In order to investigate antibody protection against viral challenge doses that may better represent those encountered in human heterosexual exposure, we utilized a low-dose repeated mucosal challenge model[14,16] in which a reduced virus dose requires several challenges to infect untreated animals, but yet eventually infects all animals with a reasonable number of challenges. In this model, we could expect to observe benefit provided by antibody if the number of challenges required for infection in treated animals was greater than the number of challenges required for infection in controls.

The human monoclonal antibody b12 neutralizes a broad range of HIV isolates from a variety of clades[17,18] through recognition of a conserved epitope overlapping the CD4-binding site of gp120[19]. A high serum concentration of b12, corresponding to about 75-fold the IC_{90} in a PBMC assay and 3,000-fold the IC_{50} in a pseudotyped virus assay provided 90% protection against a high-dose vaginal challenge with $SHIV_{SF162P3}$[20]. In addition in that study, the importance for protection of the interaction of b12 with Fc receptors was established by comparison of b12 and engineered b12 variants[20].

Here, we explored the question of whether a relatively low b12 neutralizing antibody titer could provide benefit to macaques in the low-dose repeated challenge model and simultaneously compared protection by the effector function-deficient b12 variant LALA. Based on earlier studies [14,16,21], we began the experiment with repeated 3 $TCID_{50}$ $SHIV_{162P3}$ vaginal challenge. With only a single animal infected after 11 challenges, we increased the viral dose to 10 $TCID_{50.}$ This dose corresponds to approximately 2.65×10^6 viral RNA (vRNA) copies, an amount somewhat higher than typically found in human

semen during acute infection[12,13] but substantially lower than traditional high-dose challenges with $SHIV_{SF162P3}$[14].

The study involved a total of 14 animals, consisting of 4 isotype control-treated animals, 5 animals receiving wild-type b12, and 5 animals receiving the LALA variant, which has similar neutralizing activity as b12 but does not mediate Fc effector functions [20]. Animals were *i.v.*-treated weekly (Thursday) with 1 mg/kg of antibody to maintain serum levels, based on previously reported half-lives[20]. This dose of b12 antibody is far less than the 25 mg/kg dose that provides 90% protection against high-dose challenge with $SHIV_{SF162P3}$ [20] and provides negligible protection against high-dose challenge with $SHIV_{SF162P4}$[10]. Intravaginal challenges were administered twice weekly (Friday and Monday) and blood drawn regularly to monitor viral infection, passively transferred antibody levels and serum neutralizing activity. Supplementary Figure 1 details the entire treatment course for each animal and Supplementary Table 1 summarizes antibody treatments, viral challenges, detection and day-of-peak viremia in plasma.

As shown in Figure 1 notably more challenges were required to infect b12-treated than control animals and also suggests that somewhat fewer challenges may be required to infect LALA variant-treated than wild-type b12-treated animals. One animal (b12-treated, BF68) remained uninfected after 40 consecutive 10 $TCID_{50}$ challenges. We investigated the magnitude of protection using three approaches. First, we used an adapted Kaplan-Meier analysis (Fig. 2) in which the percent of animals remaining uninfected is plotted against the number of 10 $TCID_{50}$ viral challenges. To prevent positive bias, we also included the animal BK10 that was infected in the 3 $TCID_{50}$ challenge series as if it was infected by the first 10 $TCID_{50}$ challenge (see above and Suppl. Fig. 2). The three survival curves are significantly different ($p=0.0377$). A comparison of the individual pairs of Kaplan-Meier curves reveals that LALA is significantly different from control ($p=0.0027$) while a (borderline) non-significant difference for b12 versus control ($p=0.056$) is seen due to the strong penalty incurred by including BK10 in the analysis. The same analysis excluding BK10 would indicate a significant difference ($p=0.0058$). The LALA and b12 groups did not differ significantly from each other. Second, we calculated hazard ratios for b12 and LALA-treated animals with a Cox-proportional hazard model that estimates the relative risk of infection for each of the treatment groups versus controls. Treatment with b12 was found to reduce the infection risk by about 20-fold. The risk reduction for LALA treatment was approximately 10-fold (Table 2a). Third, we calculated the reduction in infection susceptibility as described by Regoes, et al [22] by tallying the total number of 10 $TCID_{50}$ virus challenges required to infect all animals within each group (within the limits of the experiment). As shown in Table 2b b12-treated animals ($p=0.0016$) as well as LALA-treated animals ($p=0.0145$) only became infected after a significantly larger number of challenges compared to the control group. It should be noted that this number is underestimated for b12 in this type of analysis, as one b12-treated animal remained uninfected at the end of the experiment. Overall, our analyses suggest that there is a significant difference in the protection afforded by the repeated administration of 1 mg/kg of both b12 antibody and LALA variant as compared to treatment with the isotype control antibody. The approximately two-fold difference in b12 and LALA hazard ratios and the observation that

b12-treated animals resisted nearly twice as many challenges as LALA-treated animals (104 versus 61) reflects the trend previously described in a high-dose virus challenge for the effector function-crippled LALA variant to be less effective in protection than the fully effector function-competent wild type b12 antibody[20]. An analysis of peak viremias suggests a trend towards lower peak viremias in the b12-treated group compared to controls although this difference does not achieve significance (Suppl. Fig. 3). However, there is a significant difference ($p = 0.016$), about 2 orders of magnitude, between peak viremias in the b12 and LALA–treated animals, again consistent with an impact of effector function on anti-viral activity.

We determined antibody serum concentrations throughout the course of the experiment by ELISA (Supplementary Fig. 4). Considerable variations in individual serum concentration were found, but no significant correlation was found between average concentration and the number of challenges to infection. Likewise, the appearance of infection did not correlate with the magnitude of the antibody concentration at the estimated time of infection (10 – 17 days prior to detection of virus). Neutralizing antibody titers in sera were assessed in a pseudovirus assay and were as expected based on previous studies[10,20] given the antibody concentrations measured by ELISA (Supplementary Table 2). Average b12 concentrations for challenges not resulting in infection was relatively low, about 40 μg/ml, corresponding to an average 1:200 IC_{50} titer in a pseudovirus assay and to an estimated 1:5 IC_{90} titer in a PBMC assay (Table 1). MHC genotyping revealed that there was no apparent correlation with the allelic profiles of the animals in this study that would account for any unusual ability to resist infection (Supplementary Table 3).

In summary, we have shown that neutralizing antibody can provide clear benefit against repeated low-dose SHIV challenge in the macaque model at low serum antibody concentrations corresponding to modest neutralization titers. There is a concern that low-dose challenge models may be “lowering the bar” too much in terms of the requirements for protection. In this context, we note that oral chemoprophylaxis is possibly less, and certainly not more, protective against $SHIV_{SF162P3}$ challenge in the low-dose repeated challenge model, arguing that the model is not intrinsically and universally more susceptible to protective intervention[21]. If translated into protection against HIV infection in humans, the findings are a promising development for HIV vaccine design. Serum neutralizing antibody titers in the approximate range of 1:200 (IC_{50} values in a pseudovirus assay) corresponding to about 1:5 (IC_{90} values in a PBMC assay) increased the number of low-dose challenges to achieve infection here by at least an order of magnitude. If vaccination in humans led to a similar decrease of transmission rate, then one might expect a significant impact on the pandemic. Neutralizing titers above are near or below those described in the sera of a significant proportion of HIV-infected donors against multiple isolates from different clades[23–27] suggesting that such titers may be achieved with appropriate immunogens. Finally, the data further support the contribution of effector function in antibody resistance to HIV infection, underscoring the notion that the ability of an immunogen to elicit extra-neutralizing antibody activities in addition to neutralization should be assessed in vaccine evaluation.

METHODS

Macaques

All protocols for female Indian rhesus macaques were reviewed and approved by the Institutional Animal Care and Use Committees. The animals were housed in accordance with the American Association for Accreditation of Laboratory Animal Care Standards. At the start of all experiments, all animals were experimentally naïve and were negative for antibodies against HIV-1, SIV, and type D retrovirus. Virus challenge and *i.v.* antibody protocols are more fully described elsewhere[10].

Challenge virus

The virus used in this study was $SHIV_{SF162P}$ passage 3, which has been described elsewhere[28,29]. $SHIV_{SF162P3}$ retains the R5 phenotype of $HIV\text{-}1_{SF162}$. $SHIV_{SF162P3}$.propagated in phytohemagglutin (PHA)-activated rhesus macaque peripheral blood mononuclear cells (PBMC), was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Cat. No. 6526; Contributors: Drs. Janet Harouse, Cecilia Cheng-Mayer, and Ranajit Pal).

b12 and variant antibody LALA

IgG1 b12 is a human antibody (IgG1, κ) that recognizes an epitope overlapping the CD4 binding site of gp120[17,19]. Variants of b12 were created by site-directed mutagenesis as previously described[30].

Antibody production

Recombinant IgG1 (wild type b12, isotype control, and b12 LALA variant (L234A, L235A) were expressed in Chinese hamster ovary (CHO-K1) cells in glutamine-free custom formulated Glasgow minimum essential medium (GMEM Selection Media) (MediaTech Cellgro) [10]. The isotype control antibody DEN3, an anti-Dengue NS1 human IgG1 antibody, was used in this study. For large-scale tissue culture, media was supplemented with 3.5% Ultra Low Bovine IgG Fetal Bovine Serum (Invitrogen) and grown in 10-layer Cellstacks and Cell Cubes (Corning). Antibodies were purified using Protein A affinity matrix (GE Healthcare), and dialyzed against phosphate-buffered saline (PBS). Care was taken to minimize endotoxin contamination, which was monitored using a quantitative chromagenic Limulus Amoebecyte Lysate assay (Cambrex) performed according to the manufacturer's recommendations. Antibody used for the passive transfer experiments contained <1 IU of endotoxin mg^{-1}.

Plasma viral loads

The quantity of SIV viral RNA genomic copy equivalents (vRNA copy Eq/ml) in EDTA-anti-coagulated plasma was determined using a quantitative reverse-transcription PCR (QRT-PCR) assay as previously described[32]. Briefly, vRNA was isolated from plasma using a GuSCN-based procedure as described[31]. QRT-PCR was performed using the SuperScript III Platinum® One-Step Quantitative RT-PR System (Invitrogen, Carlsbad, CA). Reaction mixes did not contain bovine serum albumin (BSA). Reactions were run on a Roche

LightCycler 2.0 instrument and software. vRNA copy number was determined using LightCycler 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*.

ELISA

b12 and variant antibody concentrations in macaque sera were determined by ELISA against recombinant monomeric HIV-1 $gp120_{JR\text{-}FL}$ (kindly provided by Progenics) and is fully described elsewhere[10].

Neutralization assays

Neutralization titers in animal sera were reported by Monogram Biosciences after preparation of an HIV-1 envelope pseudotyped luciferase $SHIV_{SF162P3}$ capable of single-round replication and performed as previously described[35].

MHC genotyping

MHC genotyping by sequence-specific PCR was performed by the University of Wisconsin Genotyping Core with support of NIH grant 5R24RR16038-6 awarded to David I. Watkins and previously described [34].

Statistics

The isotype control groups consisted of a total of 4 animals (n=4), and each of the treated groups consisted of 5 animals (n=5). Statistical analyses were performed using Graph Pad Prism for Mac Software, version 5.0a (Graph Pad Software Inc., San Diego, CA, 2005). A Kaplan-Meier Survival Analysis was performed for Figure 2. The alpha level was 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mascola JR. Defining the protective antibody response for HIV-1. Curr Mol Med. 2003; 3:209–216. [PubMed: 12699358]
2. Wawer MJ, et al. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. J Infect Dis. 2005; 191:1403–1409. [PubMed: 15809897]
3. Hollingsworth TD, Anderson RM, Fraser C. HIV-1 transmission, by stage of infection. J Infect Dis. 2008; 198:687–693. [PubMed: 18662132]
4. Royce RA, Sena A, Cates W Jr, Cohen MS. Sexual transmission of HIV. N Engl J Med. 1997; 336:1072–1078. [PubMed: 9091805]

5. Gauduin MC, et al. Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1. Nat Med. 1997; 3:1389–1393. [PubMed: 9396610]

6. Shibata R, et al. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat Med. 1999; 5:204–210. [PubMed: 9930869]

7. Mascola JR, et al. Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol. 1999; 73:4009–4018. [PubMed: 10196297]

8. Mascola JR, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med. 2000; 6:207–210. [PubMed: 10655111]

9. Baba TW, et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat Med. 2000; 6:200–206. [PubMed: 10655110]

10. Parren PW, et al. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J Virol. 2001; 75:8340–8347. [PubMed: 11483779]

11. Nishimura Y, et al. Determination of a statistically valid neutralization titer in plasma that confers protection against simian-human immunodeficiency virus challenge following passive transfer of high-titered neutralizing antibodies. J Virol. 2002; 76:2123–2130. [PubMed: 11836389]

12. Chakraborty H, et al. Viral burden in genital secretions determines male-to-female sexual transmission of HIV-1: a probabilistic empiric model. AIDS. 2001; 15:621–627. [PubMed: 11317000]

13. Pilcher CD, et al. Brief but efficient: acute HIV infection and the sexual transmission of HIV. J Infect Dis. 2004; 189:1785–1792. [PubMed: 15122514]

14. Otten RA, et al. Multiple vaginal exposures to low doses of R5 simian-human immunodeficiency virus: strategy to study HIV preclinical interventions in nonhuman primates. J Infect Dis. 2005; 191:164–173. [PubMed: 15609225]

15. Klasse PJ, Shattock RJ, Moore JP. Which topical microbicides for blocking HIV-1 transmission will work in the real world? PLoS Med. 2006; 3:e351. [PubMed: 16903780]

16. Kim CN, et al. Repetitive exposures with simian/human immunodeficiency viruses: strategy to study HIV pre-clinical interventions in non-human primates. J Med Primatol. 2006; 35:210–216. [PubMed: 16872284]

17. Burton DR, et al. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science. 1994; 266:1024–1027. [PubMed: 7973652]

18. Binley JM, et al. Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J Virol. 2004; 78:13232–13252. [PubMed: 15542675]

19. Zhou T, et al. Structural definition of a conserved neutralization epitope on HIV-1 gp120. Nature. 2007; 445:732–737. [PubMed: 17301785]

20. Hessell AJ, et al. Fc receptor but not complement binding is important in antibody protection against HIV. Nature. 2007; 449:101–104. [PubMed: 17805298]

21. Subbarao S, et al. Direct stringency comparison of two macaque models (single-high vs. repeat-low) for mucosal HIV transmission using an identical anti-HIV chemoprophylaxis intervention. J Med Primatol. 2007; 36:238–243. [PubMed: 17669212]

22. Regoes RR, Longini IM, Feinberg MB, Staprans SI. Preclinical assessment of HIV vaccines and microbicides by repeated low-dose virus challenges. PLoS Med. 2005; 2:e249. [PubMed: 16018721]

23. Dhillon AK, et al. Dissecting the neutralizing antibody specificities of broadly neutralizing sera from HIV-1 infected donors. J Virol. 2007; 81:6548–6562. [PubMed: 17409160]

24. Li Y, et al. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. Nat Med. 2007; 13:1032–1034. [PubMed: 17721546]

25. Sather DN, et al. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. J Virol. 2009; 83:757–769. [PubMed: 18987148]
26. Doria-Rose NA, et al. Frequency and phenotype of human immunodeficiency virus envelope-specific B cells from patients with broadly cross-neutralizing antibodies. J Virol. 2009; 83:188–199. [PubMed: 18922865]
27. Li Y, et al. Analysis of neutralization specificities in polyclonal sera derived from human immunodeficiency virus type 1-infected individuals. J Virol. 2009; 83:1045–1059. [PubMed: 19004942]
28. Harouse JM, et al. Mucosal transmission and induction of simian AIDS by CCR5-specific simian/human immunodeficiency virus SHIV(SF162P3). J Virol. 2001; 75:1990–1995. [PubMed: 11160699]
29. Tan RC, Harouse JM, Gettie A, Cheng-Mayer C. In vivo adaptation of SHIV(SF162): chimeric virus expressing a NSI, CCR5-specific envelope protein. J Med Primatol. 1999; 28:164–168. [PubMed: 10593481]
30. Hezareh M, Hessell AJ, Jensen RC, van de Winkel JG, Parren PW. Effector function activities of a panel of mutants of a broadly neutralizing antibody against human immunodeficiency virus type 1. J Virol. 2001; 75:12161–12168. [PubMed: 11711607]
31. Lifson JD, et al. Transient early post-inoculation anti-retroviral treatment facilitates controlled infection with sparing of CD4+ T cells in gut-associated lymphoid tissues in SIVmac239-infected rhesus macaques, but not resistance to rechallenge. J Med Primatol. 2003; 32:201–210. [PubMed: 14498980]
32. Friedrich TC, et al. Subdominant CD8+ T-cell responses are involved in durable control of AIDS virus replication. J Virol. 2007; 81:3465–3476. [PubMed: 17251286]
33. Yant LJ, et al. The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J Virol. 2006; 80:5074–5077. [PubMed: 16641299]
34. Kaizu M, et al. Molecular typing of major histocompatibility complex class I alleles in the Indian rhesus macaque which restrict SIV CD8+ T cell epitopes. Immunogenetics. 2007; 59:693–703. [PubMed: 17641886]
35. Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci U S A. 2003; 100:4144–4149. [PubMed: 12644702]

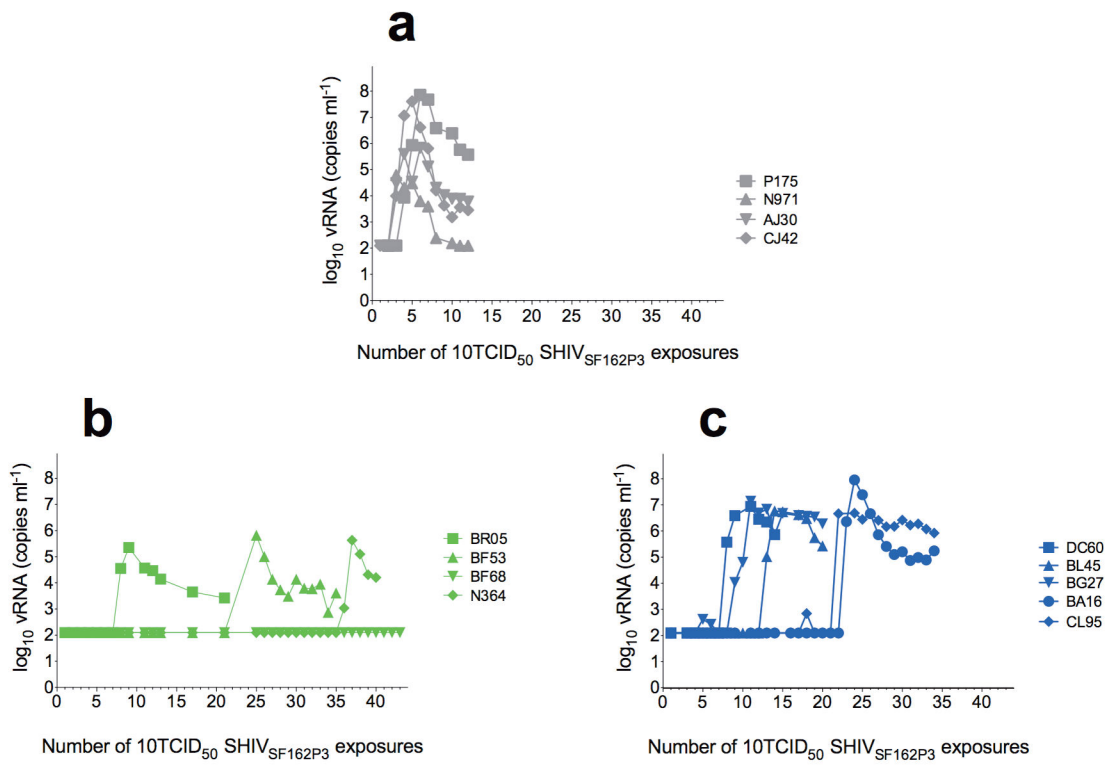

Figure 1. Protection by b12 and variant LALA during vaginal low-dose repeated challenge with $SHIV_{SF162P3}$

Female Indian rhesus macaques were treated weekly with 1 mg kg^{-1} of either b12 or b12 effector function variant LALA or an isotype control antibody (anti-dengue, DEN3) and challenged vaginally twice weekly. The viral challenge dose began at 3 $TCID_{50}$ and was subsequently increased to 10 $TCID_{50}$ $SHIV_{SF162P3}$. **a,** All animals in the isotype control groups became virus positive after a maximum of 4 challenges of 10 $TCID_{50}$. 4 out of 4 animals were infected after a total number of 10 challenges of 10 $TCID_{50}$. **b**, 3 b12-treated animals were virus positive after 6, 23, and 38 viral challenges of 10 $TCID_{50}$, respectively, and 1 animal (BF68) remained virus negative after 40 challenges. 3 out of 4 animals were infected after a total number of 107 challenges of 10 $TCID_{50}$. BK10 was infected after 6 challenges of 3 $TCID_{50}$. (See Suppl. Fig. 2.) **c,** Plasma virus was observed in the LALA-treated animals following 6, 8, 12, 17, and 23 viral challenges, respectively. 5 out of 5 animals were infected after a total of 66 challenges of 10 $TCID_{50}$. Viral challenges and *i.v.* antibody treatments were suspended after positive detection of virus in plasma but the course of infection was monitored for several weeks. The SIV viral RNA (vRNA) assay detection limit is 125 copies ml^{-1} (log 2.1).

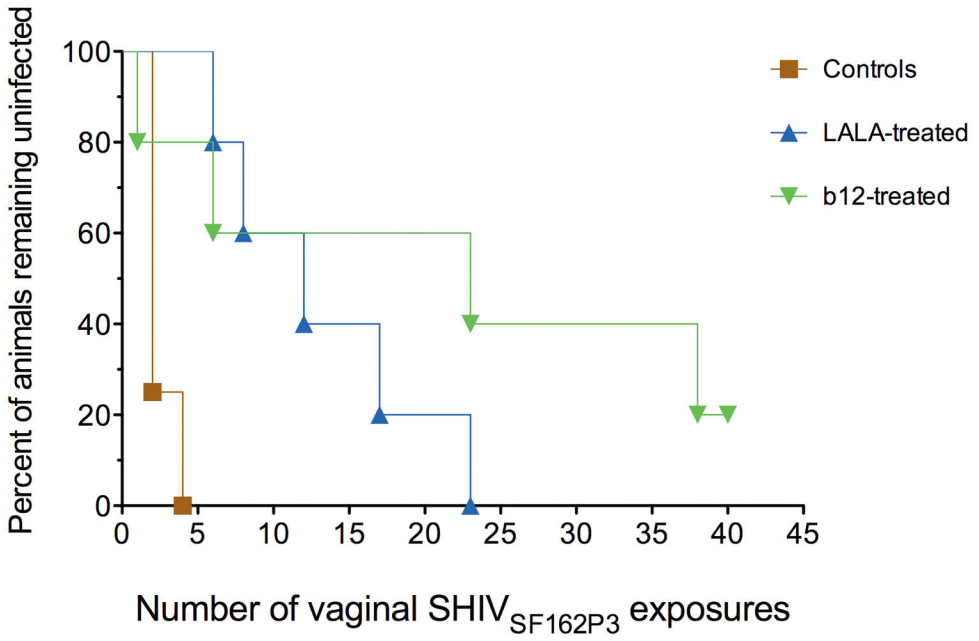

Figure 2. Kaplan-Meier analysis and magnitude of protection in low-dose (10 TCID$_{50}$) repeated challenge by b12 and LALA treatment

a. Kaplan-Meier analysis. The percent of animals remaining uninfected is plotted against the number of 10 TCID$_{50}$ viral challenges (compare Fig. 1). A single animal (BK10; b12-treated) became infected during the initial repeat 3 TCID$_{50}$ challenge (see Suppl. Fig. 2). To allow inclusion of this animal in the analysis, it is included as if it was infected in the first 10 TCID$_{50}$ challenge. The Kaplan-Meier survival curves are significantly different from each other ($p = 0.0377$; Log-rank (Mantel-Cox) test)

b. The reduction in infection susceptibility by b12 and LALA treatment is estimated by counting the number of challenges that did or did not result in infection. Again the animal BK10 is included in this analysis as being infected in the first 10 TCID$_{50}$ challenge. Both b12 ($p=0.0016$) and LALA ($p=0.0145$) are significantly different from the control (Fisher's exact test).

Table 1

Average serum antibody concentrations and neutralization titers in macaques repeatedly challenged with a low dose of $SHIV_{162P3}$ in the period before they became infected.

Animal	# of *i.v.* Ab treatments without infection	Average serum Ab [μg/ml][1]	Average IC_{50} pseudovirus assay[2]	Average IC_{90} PBMC assay[3]
LALA-treated				
DC60	9	25	1:125	1:3
BG27	11	26	1:130	1:3
BL45	13	46	1:230	1:6
CL95	15	33	1:165	1:4
BA16	18	37	1:185	1:5
b12-treated				
BK10	5	31	1:155	1:4
BR05	9	25	1:125	1:3
BF53	19	60	1:300	1:8
N364	27	53	1:265	1:7
BF68[4]	28	40	1:200	1:5

[1] Average serum concentration of transferred b12 and LALA for each macaque prior to infection.

[2] Average neutralization titer estimated from the average serum concentrations and b12 and LALA IC_{50} values in the pseudovirus assay (=0.2 μg/ml).

[3] Average neutralization titer estimated from the average serum antibody concentration and b12 and LALA IC_{90} values in a PBMC-based assay (=8 μg/ml).

[4] BF68 did not become infected after 40 challenges at 10 $TCID_{50}$.

Table 2

Statistical analyses comparing relative risk of infection between treatment groups

(**a**) The hazard ratios for b12 and LALA-treated animals are calculated using a Cox-proportional hazard model. It shows that b12 and LALA treatment significantly reduced the risk of infection at each challenge by a factor of 21 and 10 times, respectively. (**b**) The reduction in infection susceptibility [22] is also demonstrated by comparing the total number of challenges resulting in infection to the total number of challenges not leading to infection.

a Cox-proportional hazard model		
Group	***Hazard ratio***	***95% CI of ratio***
b12 vs control	21.3*	1.7; 260.9
LALA vs control	10.1	1.0; 101.0

b Infection susceptibility		
Group	***Number of 10 $TCID_{50}$ challenges leading to infection***	***Number of 10 $TCID_{50}$ challenges not leading to infection***
Control	4	6
b12	4*	104
LALA	5	61

*To prevent positive bias, BK10 has been included in this analysis as if it was infected in first challenge.