

# Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques

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**Neutralizing antibodies are thought to be crucial for HIV vaccine protection, but studies in animal models suggest that high antibody concentrations are required<sup>1</sup>. This is a major potential hurdle for vaccine design. 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<sup>2–4</sup>. Therefore, animal studies may have provided an overestimate of the levels of antibodies required for protection in humans. We investigated whether plasma concentrations of antibody corresponding to relatively modest neutralization titers *in vitro* could protect macaques from repeated intravaginal exposure to low doses of a simian immunodeficiency virus–HIV chimera (SHIV) that uses the CC chemokine receptor 5 (CCR5) co-receptor. An effector function–deficient variant of the neutralizing antibody was also included. The results show that a substantially larger number of challenges is required to infect macaques treated with neutralizing antibody than control antibody–treated macaques, and support the notion that effector function may contribute to antibody protection. Overall, the results imply that lower amounts of antibody than previously considered protective may provide benefit in the context of typical human exposure to HIV-1.**

Much of what researchers 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<sup>5–11</sup>, including intravenous (i.v.), vaginal and rectal challenge in macaques. The hallmark of most of these studies is that 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<sup>10,11</sup>. 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 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 that all control macaques 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. Transmission frequencies on the order of 1 per 1,000 coital acts have been reported in chronic infection of the donor<sup>2–4,12</sup>, increasing by about an order of magnitude in acute infection<sup>2,3,12,13</sup>. The amount of virus contained in a typical macaque challenge, albeit estimated by quantitative PCR rather than infectivity, is much higher than what would be found, for example, in the semen ejaculate of an acutely infected man<sup>12–15</sup>. Indeed, viral inoculums average  $5 \times 10^5$  copies per ejaculate, with a reported maximum of about  $2 \times 10^7$  copies<sup>12</sup>, whereas we measured the inoculum of 300 median tissue culture infectious doses (300TCID<sub>50</sub>) of SHIV<sub>SF162P3</sub>, as used in a typical high-dose macaque challenge experiment, to contain about  $1 \times 10^8$  viral copies.

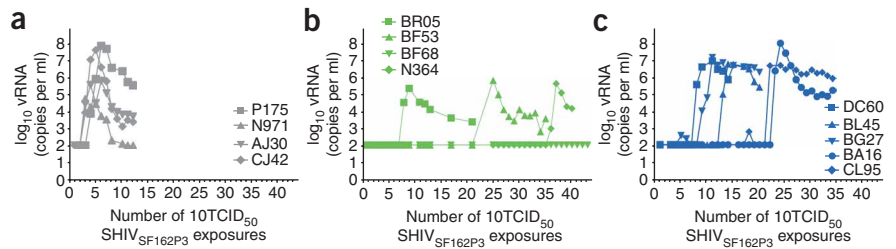
To investigate antibody protection against viral challenge doses that may better represent those encountered in human heterosexual exposure, we used a low-dose repeated mucosal challenge model<sup>14,16</sup> in which a low virus dose requires several challenges to infect untreated macaques but eventually infects all animals. With this model, we would expect to observe benefit provided by antibody if the number of challenges required for infection in treated macaques 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<sup>17,18</sup> through recognition of a conserved epitope overlapping the CD4-binding site of gp120 (ref. 19). A high serum concentration of b12, corresponding to about 75-fold the concentration required to inhibit 90% of viral replication *in vitro* (IC<sub>90</sub>) in a peripheral blood mononuclear cell

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**Figure 1** Viral loads in antibody-treated macaques during vaginal low-dose repeated challenge with SHIV<sub>SF162P3</sub>. (a–c) Plasma viral loads assessed by the measurement of SIV viral RNA using a quantitative reverse-transcription PCR (QRT-PCR) assay. The quantity of SIV viral RNA genomic copies (vRNA copies per ml) is plotted against the total number of viral challenges at a dose of 10TCID<sub>50</sub>. All macaques in each antibody treatment group were treated with 1 mg per kg body weight and are shown separately. (a) Macaques treated with the isotype control antibody. (b) Macaques treated with b12. One b12-treated macaque (BF68) remained virus negative after 40 challenges. One b12-treated macaque (BK10) was infected after six challenges of 3TCID<sub>50</sub> and was therefore not presented in these data (Supplementary Fig. 2). (c) Macaques treated with b12 LALA variant. 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 per ml (log 2.1). The number of challenges is depicted here; the time course of challenge was interrupted in some instances as depicted in detail in Supplementary Figure 1.



(PBMC) assay and 3,000-fold the IC<sub>50</sub> in a pseudotyped virus assay, provided 90% protection against a high-dose vaginal challenge with SHIV<sub>SF162P3</sub><sup>20</sup>. In the same study, the importance of the interaction of b12 with Fc receptors for protection was established by comparison of b12 and engineered b12 variants<sup>20</sup>.

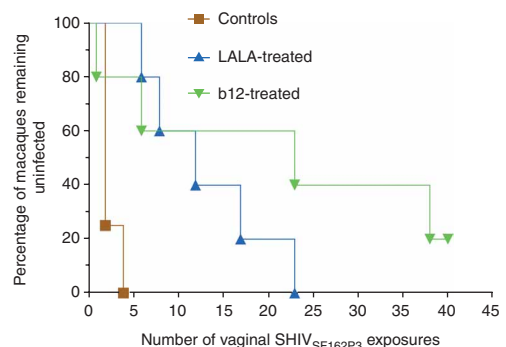
Here we explored the question of whether a relatively low b12 serum neutralizing antibody titer could provide benefit to macaques in the low-dose repeated challenge model and simultaneously compared protection by the effector function-deficient b12 variant LALA. On the basis of earlier studies<sup>14,16,21</sup>, we began the experiment with repeated vaginal challenges using an inoculum dose of 3TCID<sub>50</sub> of SHIV<sub>162P3</sub>. With only a single macaque infected after 11 challenges, we increased the viral dose to 10TCID<sub>50</sub>. This dose corresponds to approximately  $2.65 \times 10^6$  viral RNA (vRNA) copies, an amount somewhat larger than typically found in human semen during acute infection<sup>12,13</sup> but only a few percent (2–3%) of traditional high-dose challenges with SHIV<sub>SF162P3</sub><sup>20</sup>.

The study involved a total of 14 macaques, consisting of four isotype control-treated macaques, five macaques receiving wild-type b12 and five macaques receiving the LALA variant, which has similar neutralizing activity as b12 but does not mediate Fc effector functions<sup>20</sup>. To maintain serum levels of antibody, we treated macaques i.v. weekly with 1 mg of b12 or LALA per kg body weight, on the basis of previously reported half-lives<sup>20</sup>. This dose of b12 antibody is far less than the 25 mg per kg body weight dose that provided 90% protection against high-dose challenge with SHIV<sub>SF162P3</sub><sup>20</sup>. Further, 1 mg per kg body weight of b12 provided negligible protection against high-dose challenge with SHIV<sub>SF162P4</sub><sup>10</sup>. Intravaginal challenges were administered twice weekly, and blood was 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, number of viral challenges, day of viremia detection and day-of-peak viremia in plasma.

The results show overall that notably more challenges were required to infect b12-treated macaques compared to control macaques (Fig. 1), and they also suggest that somewhat fewer challenges may be required to infect LALA variant-treated macaques compared to wild-type b12-treated macaques. Macaques in the isotype control group became virus positive after a maximum of four challenges of 10TCID<sub>50</sub> (Fig. 1 and Supplementary Table 1). A cumulative total number of ten challenges of 10TCID<sub>50</sub> was sufficient to infect all four control macaques (Supplementary Table 1). In the b12-treated group, macaque BK10 was infected after six challenges of 3TCID<sub>50</sub> (Supplementary Fig. 2). After we switched to the 10TCID<sub>50</sub> challenge regime, three b12-treated macaques became virus positive

after 6, 23 or 38 viral challenges, and one macaque (BF68) remained virus negative after 40 challenges (Fig. 1 and Supplementary Table 1). Thus three of these four b12-treated macaques were infected by a cumulative total of 107 challenges of 10TCID<sub>50</sub>. In the LALA-treated group, plasma virus was observed after 6, 8, 12, 17 or 23 viral challenges (Fig. 1 and Supplementary Table 1). The five LALA-treated macaques were thus infected by a cumulative total of 66 challenges of 10TCID<sub>50</sub> virus. We suspended viral challenges and i.v. antibody treatments after positive detection of virus in plasma, but we monitored the course of infection for several weeks.

We investigated the magnitude of protection using three approaches. First, we used an adapted Kaplan-Meier analysis (Fig. 2) in which the percentage of macaques remaining uninfected is plotted against the number of 10TCID<sub>50</sub> viral challenges. To prevent positive bias, the macaque (BK10), who was infected in the 3TCID<sub>50</sub> challenge series, was included in the analysis as if infected by the first 10TCID<sub>50</sub> challenge (Supplementary Fig. 2). The three survival curves are significantly different ( $P = 0.0377$ ; Fig. 2). A comparison of the individual pairs of Kaplan-Meier curves reveals that LALA is significantly different from the control in preventing infection ( $P = 0.0027$ ). Owing to the strong penalty incurred by including BK10 in the analysis (Fig. 2), the difference between b12 and control did not reach significance ( $P = 0.056$ ). The same analysis excluding BK10 yielded a significant difference ( $P = 0.0058$ ). The LALA and b12



**Figure 2** Kaplan-Meier analysis and magnitude of protection by b12 and LALA treatment in low-dose (10TCID<sub>50</sub>) repeated challenge. The percentage of macaques remaining uninfected is plotted against the number of 10TCID<sub>50</sub> viral challenges. A single macaque (BK10; b12-treated) became infected during the initial repeat 3TCID<sub>50</sub> challenge. To allow inclusion of this macaque in the analysis, we included as if it was infected in the first 10TCID<sub>50</sub> challenge. The Kaplan-Meier survival curves are significantly different from each other ( $P = 0.0377$ ; log-rank (Mantel-Cox) test).

**Table 1 Statistical analyses comparing relative risk of infection between treatment groups**

(a) Group	Cox proportional hazard model	
	Hazard ratio	95% CI of ratio
b12 versus control	21.3 <sup>a</sup>	1.7; 260.9
LALA versus control	10.1	1.0; 101.0

(b) Group	Infection susceptibility	
	Number of 10TCID <sub>50</sub> challenges leading to infection	Number of 10TCID <sub>50</sub> challenges not leading to infection
Control	4	6
b12	4 <sup>a</sup>	104
LALA	5	61

(a) The hazard ratios for b12 and LALA-treated macaques are calculated using a Cox proportional hazard model and show that b12 significantly reduced the risk of infection at each challenge by a factor of 21 ( $P = 0.0168$ ). The risk of infection at each challenge was reduced by a factor of 10 by LALA treatment, which is borderline significant ( $P = 0.0499$ ). (b) The reduction in infection susceptibility is also demonstrated by comparing the total number of challenges resulting in infection to the total number of challenges not leading to infection. Both b12 ( $P = 0.0016$ ) and LALA ( $P = 0.0145$ ) are significantly different from the control (Fisher's exact test).

<sup>a</sup>To prevent positive bias, BK10 has been included in this analysis as if this macaque was infected in the first challenge at 10TCID<sub>50</sub>.

groups did not differ significantly from each other (Fig. 2). Second, we calculated hazard ratios for b12- and LALA-treated macaques with a Cox proportional hazard model that estimates the relative risk of infection for each of the treatment groups versus the controls. We found that treatment with b12 reduced the infection risk at each challenge by a factor of 21 (Table 1a) and for LALA treatment by a factor of 10 (Table 1a). Third, we calculated the reduction in infection susceptibility as described previously<sup>22</sup> by tallying the total number of 10TCID<sub>50</sub> virus challenges required to infect all macaques within each group (within the limits of the experiment). b12-treated macaques ( $P = 0.0016$ ) as well as LALA-treated macaques ( $P = 0.0145$ ) became infected after a significantly larger number of challenges compared to the control group (Table 1b). Of note, this number is underestimated for b12 in this type of analysis, as one b12-treated macaque 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 per kg body weight of both b12 antibody and LALA variant as compared to treatment with the isotype control antibody. The approximately twofold difference in b12 and LALA hazard ratios and the observation that b12-treated macaques resisted nearly twice as many challenges as LALA-treated macaques (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<sup>20</sup>. An analysis of peak viremias suggests a trend toward lower peak viremias in the b12-treated group compared to controls, although this difference does not achieve significance (Supplementary Fig. 3). However, there is a significant difference ( $P = 0.016$ ), about two orders of magnitude, between peak viremias in the b12- and LALA-treated macaques, again consistent with an impact of effector function on antiviral activity.

We determined antibody serum concentrations throughout the course of the experiment by ELISA (Supplementary Fig. 4). We found considerable variations in individual serum concentrations, but we did not find significant correlation 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 d before

detection of virus). We assessed neutralizing antibody titers in sera in a pseudovirus assay, and they were as expected from previous studies<sup>10,20</sup>, given the antibody concentrations measured by ELISA (Supplementary Table 2). Average b12 concentrations for challenges not resulting in infection were relatively low, about 40  $\mu\text{g ml}^{-1}$ , corresponding to an average serum IC<sub>50</sub> titer of 1:200 in a pseudovirus assay (that is, serum at a 1 in 200 dilution produced 50% neutralization in the assay) and to an estimated serum IC<sub>90</sub> titer of 1:5 in a PBMC assay (Table 2). Major histocompatibility complex (MHC) genotyping revealed that there was no apparent correlation with the allelic profiles of the macaques 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 a 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<sub>SF162P3</sub> challenge in the low-dose repeated challenge model, arguing that the model is not intrinsically and universally more susceptible to protective intervention<sup>21</sup>. 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<sub>50</sub> values in a pseudovirus assay, corresponding to about 1:5 IC<sub>90</sub> values in a PBMC assay, increased the number of low-dose challenges necessary to achieve infection here by at least an order of magnitude. If vaccination in humans were to lead to a similar decrease of transmission rate, then one might expect a major impact on the pandemic. Neutralizing titers achieved in this study are near or below those described in the sera of a substantial proportion of HIV-infected donors against multiple isolates from various clades<sup>23–27</sup>, 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

**Table 2 Average serum antibody concentrations and neutralization titers in macaques repeatedly challenged with a low dose of SHIV<sub>SF162P3</sub> in the period before they became infected**

Animal	Number of i.v. antibody treatments without infection	Average serum antibody ( $\mu\text{g ml}^{-1}$ ) <sup>a</sup>	Average IC <sub>50</sub> pseudovirus assay <sup>b</sup>	Average IC <sub>90</sub> PBMC assay <sup>c</sup>
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 <sup>d</sup>	28	40	1:200	1:5

<sup>a</sup>Average serum concentration of transferred b12 and LALA for each macaque before infection.

<sup>b</sup>Average neutralization titer estimated from the average serum concentrations and b12 and LALA IC<sub>50</sub> values in the pseudovirus assay ( $=0.2 \mu\text{g ml}^{-1}$ ). <sup>c</sup>Average neutralization titer estimated from the average serum antibody concentration and b12 and LALA IC<sub>90</sub> values in a PBMC-based assay ( $=8 \mu\text{g ml}^{-1}$ ). <sup>d</sup>BF68 did not become infected after 40 challenges at 10TCID<sub>50</sub>.

immunogen to elicit extra-neutralizing antibody activities in addition to neutralization should be assessed in vaccine evaluation.

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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ELISA for recombinant monomeric HIV-1 gp120<sub>RF-FL</sub> was provided by Progenics. We thank K. Saye-Francisco for antibody production and quality control assistance at The Scripps Research Institute and S. Hoffenberg at the IAVI AIDS Vaccine Design Laboratory. We thank A. Haahr Andreasen for statistical advice and analysis. We are grateful for the assistance provided by E. Rakasz, G. Borchardt, and C. McNair with genotyping and viral load assessments at the Wisconsin National Primate Research Center. We also thank M. Huber and R. Astronomo for reviewing the manuscript. Support for this work was provided by US National Institutes of Health (NIH) grant AI55332 to D.R.B., by the Neutralizing Antibody Consortium of the International AIDS Vaccine Initiative and by the Swiss National Foundation, Fellowship PA00A-109033.

## AUTHOR CONTRIBUTIONS

Project planning was performed by A.J.H., L.H., P.A.M. and D.R.B.; experimental work was done by A.J.H., L.H., M.H. and D.M.T.; data analysis was done by A.J.H., L.H., P.P., W.K.B., P.W.H.I.P. and D.R.B.; and A.J.H., P.P., P.W.H.I.P. and D.R.B. composed the manuscript.

## COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemedicine/>.

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## ONLINE METHODS

**Macaques.** All protocols for female Indian rhesus macaques were reviewed and approved by the Institutional Animal Care and Use Committees of The Scripps Research Institute and Tulane National Primate Research Center, Tulane University. We housed the macaques in accordance with the American Association for Accreditation of Laboratory Animal Care Standards. At the start of all experiments, all macaques were experimentally naive 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<sup>10</sup>.

**Challenge virus.** In this study, we used SHIV<sub>SF162P</sub> passage 3 virus, which has been described elsewhere<sup>28,29</sup>. SHIV<sub>SF162P3</sub> retains the R5 phenotype of HIV-1<sub>SF162</sub>. We obtained SHIV<sub>SF162P3</sub> propagated in phytohemagglutinin-activated rhesus macaque PBMCs 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 and variant antibody LALA.** IgG1 b12 is a human antibody (IgG1,  $\kappa$ ) that recognizes an epitope overlapping the CD4 binding site of gp120<sup>17,19</sup>. We created variants of b12 by site-directed mutagenesis as previously described<sup>30</sup>.

**Antibody production.** We expressed recombinant IgG1 (wild-type b12, isotype control and b12 LALA variant (L234A, L235A)) in Chinese hamster ovary (CHO-K1) cells in glutamine-free, custom-formulated Glasgow minimum essential medium (GMEM Selection Media) (MediaTech Cellgro)<sup>10</sup>. We used DEN3, a dengue virus-specific NS1 human IgG1 antibody, as the isotype control antibody in this study. For large-scale tissue culture, we supplemented medium with 3.5% Ultra Low Bovine IgG Fetal Bovine Serum (Invitrogen) and grew it in ten-layer Cellstacks and Cell Cubes (Corning). We purified antibodies with Protein A affinity matrix (GE Healthcare) and dialyzed them against PBS. We took care to minimize endotoxin contamination, which we monitored by a quantitative chromagenic Limulus Amoebocyte Lysate assay (Cambrex) performed according to the manufacturer's recommendations. Antibody used for the passive transfer experiments contained <1 international unit of endotoxin per mg.

**Plasma viral loads.** We quantified SIV vRNA genomic copies in EDTA-anticoagulated plasma by a QRT-PCR assay as previously described<sup>31</sup>. Briefly, we isolated vRNA from plasma by a guanidinium thiocyanate (GuSCN-based) procedure as previously described<sup>32</sup>. We performed QRT-PCR with the SuperScript III Platinum One-Step Quantitative RT-PR System (Invitrogen). Reaction mixes did not contain BSA. We ran reactions on a Roche LightCycler 2.0 instrument and software. We determined vRNA copy number with

LightCycler 4.0 software (Roche Molecular Diagnostics) to interpolate sample crossing points onto an internal standard curve prepared from tenfold serial dilutions of a synthetic RNA transcript representing a conserved region of SIV *gag*.

**Enzyme-linked immunosorbent assay.** We determined b12 and variant antibody concentrations in macaque sera by ELISA for recombinant monomeric HIV-1 gp120<sub>JR-FL</sub> (kindly provided by Progenics), fully described elsewhere<sup>10</sup>.

**Neutralization assays.** Neutralization titers in animal sera were reported by Monogram Biosciences after preparation of an HIV-1 envelope-pseudotyped luciferase SHIV<sub>SF162P3</sub> capable of single-round replication, performed as previously described<sup>33</sup>.

**Major histocompatibility complex genotyping.** MHC genotyping by sequence-specific PCR was performed by the University of Wisconsin Genotyping Core as previously described<sup>34,35</sup>.

**Statistical analyses.** The isotype control groups consisted of a total of four macaques ( $n = 4$ ), and each of the treated groups consisted of five macaques ( $n = 5$ ). We performed statistical analyses with Graph Pad Prism for Mac, version 5.0a (Graph Pad). A Kaplan-Meier survival analysis was performed for **Figure 2**. The alpha level was 0.05.

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