

1 **Vaccine-induced protection from homologous Tier 2 simian-human**  
2 **immunodeficiency virus challenge in nonhuman primates**

3

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55 **SUMMARY:**

56 Passive administration of HIV neutralizing antibodies (nAbs) can protect macaques from  
57 hard-to-neutralize (Tier 2) chimeric simian-human immunodeficiency virus (SHIV)  
58 challenge. However, conditions for nAb-mediated protection following vaccination have  
59 not been established. Here, we selected groups of 6 rhesus macaques with either high  
60 or low serum nAb titers from a total of 78 animals immunized with recombinant native-like  
61 (SOSIP) Env trimers from the BG505 HIV isolate. Repeat intrarectal challenge with  
62 homologous Tier 2 SHIV<sub>BG505</sub> led to rapid infection in unimmunized and low-titer animals.  
63 In contrast, high-titer animals demonstrated protection that was gradually lost as nAb  
64 titers waned over weeks to months. From these results, we determined that an autologous  
65 serum ID<sub>50</sub> nAb titer of ~1:500 was required to afford over 90% protection from medium-  
66 dose SHIV infection. We further identified autologous nAb titers, but not ADCC or T cell  
67 activity, as strong correlates of protection. These results provide proof-of-concept that  
68 Env protein-based vaccination strategies can protect against hard-to-neutralize SHIV  
69 challenge in rhesus macaques by inducing Tier 2 nAbs, provided appropriate neutralizing  
70 titers can be reached and maintained.

71

72 **KEYWORDS:**

73 HIV vaccine, neutralizing antibodies, BG505, Tier 2 protection, non-human primates,  
74 ADCC

75 **INTRODUCTION:**

76 Several vaccine strategies are being pursued to stimulate protective immunity against  
77 HIV, including those that combine the elicitation of cellular and humoral responses  
78 (Haynes and Burton, 2017; Stephenson et al., 2016). One of the most intensively studied  
79 approaches is focused on inducing neutralizing antibodies (nAbs) to the virus. Early  
80 pioneering monkey studies showed that DNA/gp120-immunization induces nAb  
81 responses that can protect against Tier 1 virus challenge (Barnett et al., 2008; 2010; Pal  
82 et al., 2006). However, Tier 1 viruses like SHIV<sub>Ba-L</sub> and SHIV<sub>SF162-P4</sub> are easy to neutralize,  
83 typically lead to self-limiting infections and are not considered representative of circulating  
84 viruses in the HIV pandemic. Two recent studies investigated vaccine-induced protection  
85 from a mixed Tier SIVsmE660 swarm and attributed protection, in part, to nAb and other  
86 Ab responses (Keele et al., 2017; Roederer et al., 2015). Currently there has not been  
87 clear evidence of vaccination-induced nAbs providing protection against viruses  
88 possessing hard-to-neutralize clinically relevant Tier 2 HIV Env in humans or NHP  
89 models.

90 Enthusiasm for the nAb approach arises from the association of nAbs with  
91 protection for other viruses (Tomaras and Plotkin, 2017) and the demonstration that  
92 passively administered HIV-neutralizing monoclonal antibodies (mAbs) can afford  
93 protection in monkey and mouse models of HIV infection (Gautam et al., 2016; Gruell et  
94 al., 2013; Hessell et al., 2007; Mascola et al., 2000; Moldt et al., 2012; Parren et al., 2001;  
95 Pegu et al., 2014; Shingai et al., 2014). As HIV does not infect monkeys, HIV-neutralizing  
96 mAbs are assessed by their ability to protect against chimeric simian-human  
97 immunodeficiency virus (SHIV) challenge in rhesus macaques (*Macaca mulatta*).

98 However, a major problem in establishing vaccine-induced nAb protection in the  
99 SHIV/macaque model has been the notorious difficulty in inducing nAbs by immunization.  
100 Indeed, induction of broadly neutralizing antibodies (bnAbs) via immunization has thus  
101 far only been achieved reproducibly in cows (Sok et al., 2017). However, we recently  
102 showed reliable induction of autologous strain-specific nAbs in macaques against a hard-  
103 to-neutralize Tier 2 HIV isolate through use of well-ordered and stabilized HIV envelope  
104 glycoprotein (Env) SOSIP trimers as immunogens in optimized approaches (Pauthner et  
105 al., 2017), building on previous SOSIP immunization studies in NHPs (Havenar-Daughton  
106 et al., 2016; Sanders et al., 2015; Torrents de la Peña et al., 2017). To carry out a  
107 protection experiment in macaques then requires construction of a SHIV with the same  
108 Env sequence as the immunizing trimer. Fortunately, it has recently become possible to  
109 reliably generate infectious SHIVs using *env* sequences from most primary Tier 2 HIV  
110 strains (Del Prete et al., 2017; Li et al., 2016).

111 In this study, building upon the advances in both trimer-based immunization  
112 strategies and SHIV generation, we immunized macaques with SOSIP trimers of the  
113 BG505 *env* sequence (de Taeye et al., 2015; Kulp et al., 2017; Torrents de la Peña et al.,  
114 2017), induced BG505-specific Tier 2 nAbs, and then challenged animals intrarectally  
115 with the neutralization-resistant, pathogenic SHIV<sub>BG505</sub> (Li et al., 2016), that carries the  
116 S375Y mutation to increase infectivity in NHPs. We found that protection could be  
117 achieved and was critically dependent on the level of serum nAb titers, but not on other  
118 antibody parameters such as V3 binding titers, antibody-dependent cellular cytotoxicity  
119 (ADCC), or the induction of T cell activity. We determined an approximate threshold titer

120 for vaccine-induced protection that establishes an experimental benchmark for  
121 comparison with nAb-based vaccines to HIV-1.

122 **RESULTS:**

123 **Balanced selection of challenge animals**

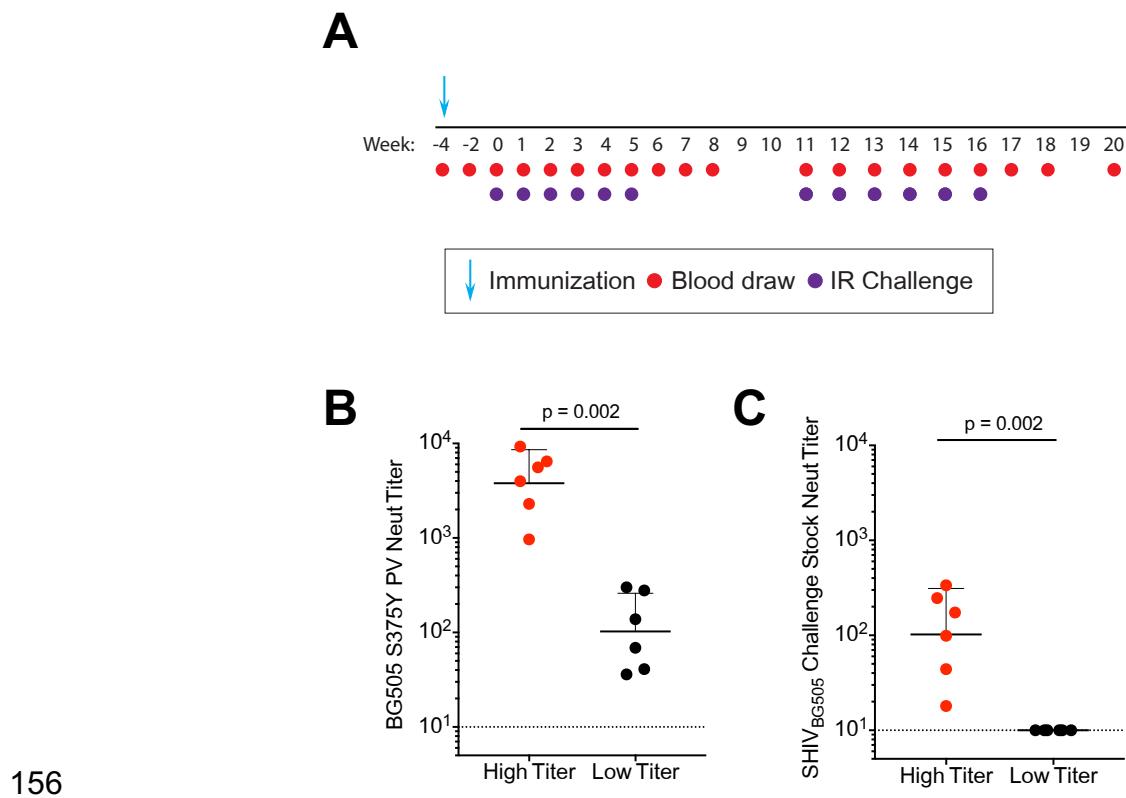
124 Our goal was to assess the capability of vaccine-elicited Tier 2 nAbs to protect from  
125 homologous Tier 2 challenge with neutralization-resistant, pathogenic SHIV<sub>BG505</sub> (Li et al.,  
126 2016). We previously developed a protocol for the reliable induction of nAbs and  
127 immunized 78 NHPs (Pauthner et al., 2017), inducing varying levels of autologous Tier 2  
128 nAb titers after three immunizations with native-like BG505 Env trimers (de Taeye et al.,  
129 2015; Kulp et al., 2017; Torrents de la Peña et al., 2017). To design a challenge study  
130 powered to detect differences between NHPs with either high or low BG505 nAb titers,  
131 we selected six NHPs that were among the top neutralizers and carefully matched them  
132 as closely as possible, in terms of gender, age and weight, with six low nAb titer animals  
133 that received similar or identical immunogens (Figure S1, A to C). We note that none of  
134 the protective or viral breakthrough or antibody kinetic effects described below could be  
135 associated with a particular immunogen; as will be seen, observed effects are primarily  
136 associated with nAb titer. We further enrolled 12 unimmunized control animals into the  
137 study. All animals were genotyped for Mamu and TRIM-5 $\alpha$  alleles associated with host  
138 restriction in non-human primates (Table S1).

139

140 **Design of the SHIV<sub>BG505</sub> challenge study**

141 To identify a challenge dose that reliably infects unimmunized control animals, we  
142 performed a pilot study by intrarectally (IR) inoculating two groups of six macaques at  
143 weekly intervals with either  $0.5 \times 10^8$  or  $1.4 \times 10^7$  virions of the SHIV<sub>BG505</sub> S375Y challenge

144 virus grown in rhesus CD4<sup>+</sup> T cells (Figure S2). For the main study, we selected a  
145 challenge dose of 1.4 x 10<sup>7</sup> virions (1ml of 1:75 diluted challenge stock), since it infected  
146 at least 4/6 animals following the 1<sup>st</sup> challenge and the remaining two animals after the  
147 2<sup>nd</sup> challenge in the pilot study. To maximize nAb titer levels in NHPs prior to challenge,  
148 high and low nAb titer animals each received a fourth immunization with the previously  
149 used immunogens, adjuvanted with an ISCOMATRIX-like saponin (Figure 1A). All NHPs  
150 responded with increased autologous nAb titers two weeks post-boost. High and low nAb  
151 titer animals continued to show significantly different geometric mean ID<sub>50</sub> titers of 1:3790  
152 and 1:103 to BG505 S375Y pseudovirus (p=0.002, Figure 1B), respectively.  
153 Neutralization titers to rhesus CD4<sup>+</sup> T-cell grown SHIV<sub>BG505</sub> S375Y challenge stock were  
154 ~30-fold lower, with significantly different geometric mean titers of 1:102 and < 1:10 when  
155 tested on TZM-bl target cells (p=0.002, Figure 1C), respectively.



157 **Figure 1. Challenge study design.** (A) Animals, except for the controls, received a  
158 booster immunization using the same immunogen that had last been used during the  
159 preceding immunization study (Pauthner et al., 2017), typically 100 $\mu$ g SOSIP trimer  
160 adjuvanted in IscoMIT. Intrarectal (IR) challenges with SHIV<sub>BG505</sub> S375Y commenced 4  
161 weeks thereafter. All groups of animals received 6 IR challenges starting at week 0. High  
162 nAb titer animals that had undetectable serum viral loads at week 6 received a second  
163 set of 6 weekly IR challenges starting week 11. (B-C) Serum neutralizing ID<sub>50</sub> titers in  
164 high and low nAb titer animals at week -2: BG505 S375Y pseudovirus (B) and rh-CD4-  
165 grown SHIV<sub>BG505</sub> S375Y challenge stock. (C). Shown are geometric means with  
166 geometric SD, significant differences were determined using two-tailed Mann-Whitney U  
167 tests.

168 **Robust protection of high nAb titer group NHPs**

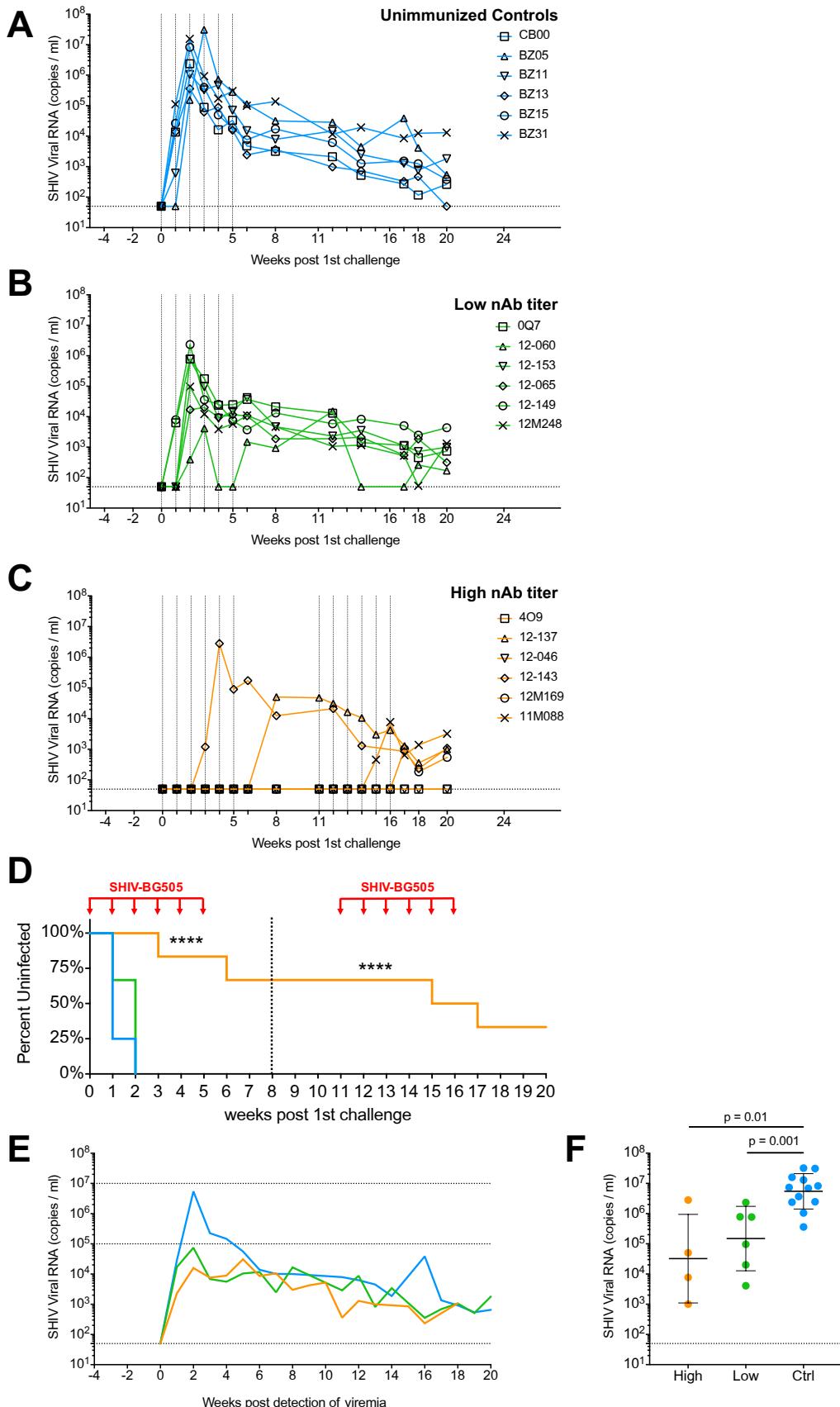
169 Four weeks after the booster immunization, all animals received six weekly IR challenges  
170 with SHIV<sub>BG505</sub>. To maximize comparability, viral loads for all animals and time points  
171 were simultaneously measured at weeks 6 and 20 (Figure 2, A to C). 5/6 concurrent  
172 unimmunized control animals were infected after the 1<sup>st</sup> challenge and the remaining  
173 animal became viremic after the 2<sup>nd</sup> challenge (Figure 2A). Combined with the  
174 unimmunized control NHPs of the dose-matched titration group (Figure S2), at least 9 of  
175 12 unimmunized animals became infected following a single challenge, which  
176 approximates to an animal infectious dose of 75% (AID<sub>75</sub>) (Table S2). Thus, the dose of  
177 1.4 x 10<sup>7</sup> SHIV<sub>BG505</sub> virions per IR inoculation employed in this study sets a relatively high  
178 bar for protection. Unimmunized control animals showed high peak viremia (geometric  
179 mean of 5.5 x 10<sup>6</sup> copies/ml) and consistent set point viral loads in the range of 9.8 x 10<sup>2</sup>  
180 – 4.7 x 10<sup>4</sup> (geometric mean of 6.2 x 10<sup>3</sup>) at 12 weeks post-infection (Figure 2, A and E,  
181 Figure S2).

182 2/6 low nAb titer animals became infected after the first challenge and the  
183 remaining four animals became viremic following the second challenge (Figure 2B),  
184 indicating that low nAb titer animals had a possible mild reduction in per-exposure risk  
185 compared to unimmunized controls, but the difference was not significant (Figure 2D,  
186 Table S3). However, low nAb titer animals had significantly lowered peak viral loads  
187 compared to unimmunized controls (1.5 x 10<sup>5</sup> vs. 5.5 x 10<sup>6</sup> copies/ml) (p = 0.001, Figure  
188 2, E and F).

189 In contrast, high nAb titer animals showed highly significant protection from  
190 challenge following the first set of challenges at week 8 (Figure 2D, Table S3). Except for

191 macaque 12-143, no animals showed viremia at week 6 and were therefore scheduled to  
192 receive a second set of six challenges starting at week 11. The goal of the second  
193 challenge set was to assess the duration of protection and to estimate a protective nAb  
194 titer threshold as nAb titers declined over time. Over the course of both challenge sets,  
195 four initially high nAb titer animals became viremic, after 3, 6, 10 and 12 virus inoculations;  
196 however, two animals showed complete sterilizing protection (Figure 2C). In addition,  
197 infected high-titer macaques showed significantly lowered peak viremia compared to  
198 unimmunized controls ( $3.2 \times 10^4$  vs  $5.5 \times 10^6$  copies/ml;  $p = 0.01$ , Figure 2, E and F),  
199 similar to the low nAb titer animals. We theorize that sub-protective levels of serum nAbs  
200 at the time of infection, as well as activation of vaccine-induced memory B cells leading  
201 to the rapid production of Abs, likely curtail emerging primary viremia, thus reducing peak  
202 viral loads.

203 The protection from infection for high nAb titer animals was highly significant  
204 compared to unimmunized controls after both 6 and 12 challenges ( $p < 0.0001$ , Figure  
205 2D, Table S3) and animals in this group remained uninfected for a median of 11  
206 challenges (Table S3). It should be emphasized that for all vaccinated animals, nAb titers  
207 declined throughout the challenge schedule, unless animals became infected as detailed  
208 below. In this respect, our study distinguishes itself from those in which antibody titers  
209 leveled off prior to challenge, as a result of the short 4-week interval here between final  
210 immunization and first challenge. However, we deliberately took advantage of declining  
211 nAb titers to determine a nAb-mediated threshold of protection.



213 **Figure 2. High nAb titer animals show robust protection.** Viral loads of animals  
214 throughout the challenge schedule: unimmunized concurrent controls (**A**), low nAb titer  
215 (**B**) and high nAb titer macaques (**C**). IR challenges are indicated with vertical dotted  
216 lines. Horizontal dotted lines denote the limit of detection. (**D**) Kaplan-Meier curves  
217 indicating percent uninfected animals over the duration of the study. Challenge time  
218 points are indicated with red arrows. The high nAb titer group infection-rate was  
219 statistically different from the low nAb titer and unimmunized control groups. \*\*\* =  $p <$   
220 0.0001. Statistics were calculated for both the first (dotted line at week 8) and second  
221 challenge sets (see Table S3) (**E**) Geometric mean viral loads of indicated groups,  
222 normalized to the detection of viremia in the blood. Horizontal lines at  $10^5$  and  $10^7$  viral  
223 RNA copies/ml serve as visual aids. (**F**) Comparison of peak viral loads between high  
224 nAb titer (high), low nAb titer (low), and unimmunized (ctrl) animals. Geometric mean  
225 titers are shown with geometric standard deviations. Significant differences were  
226 determined using two-tailed Mann-Whitney U tests.

227

228 **Tier 2 nAb titers correlate with protection**

229 Unimmunized control animals developed BG505 S375Y pseudovirus ID<sub>50</sub> nAb titers 8-12  
230 weeks postinfection in response to SHIV<sub>BG505</sub> S375Y infection (Figure 3A). By  
231 comparison, vaccine-induced nAb titers in low titer animals initially declined, but then  
232 began to rise only 1-2 weeks postinfection, i.e. much more rapidly than in unimmunized  
233 animals (Figure 3B). The early rise of nAb titers following infection of low nAb titer animals  
234 is thus likely due to recall responses of BG505 Env immunogen-induced memory B cells.

235 Interestingly, BG505 nAb titers rose to substantially higher ID<sub>50</sub> titers (3/6 animals >1:750)  
236 than previously achieved by four immunizations of these six animals with ISCOMs-  
237 adjuvanted BG505 native-like Env trimers (Figures 1B, 3B, S1B). The marked increases  
238 in BG505 nAb titers following infection suggest that outbred macaques that did not  
239 respond well to vaccination were not inherently incapable, by genetic or other means, of  
240 developing high nAb titer responses, although this conclusion should be caveated by the  
241 observation that antigen dose and delivery vary greatly between vaccination and natural  
242 infection. Better immunogen presentation and more targeted adjuvants are likely needed  
243 to increase the reliability of high nAb titer development, and to address current  
244 shortcomings in the durability of nAb responses induced by protein-only immunizations  
245 (Havenar-Daughton et al., 2017).

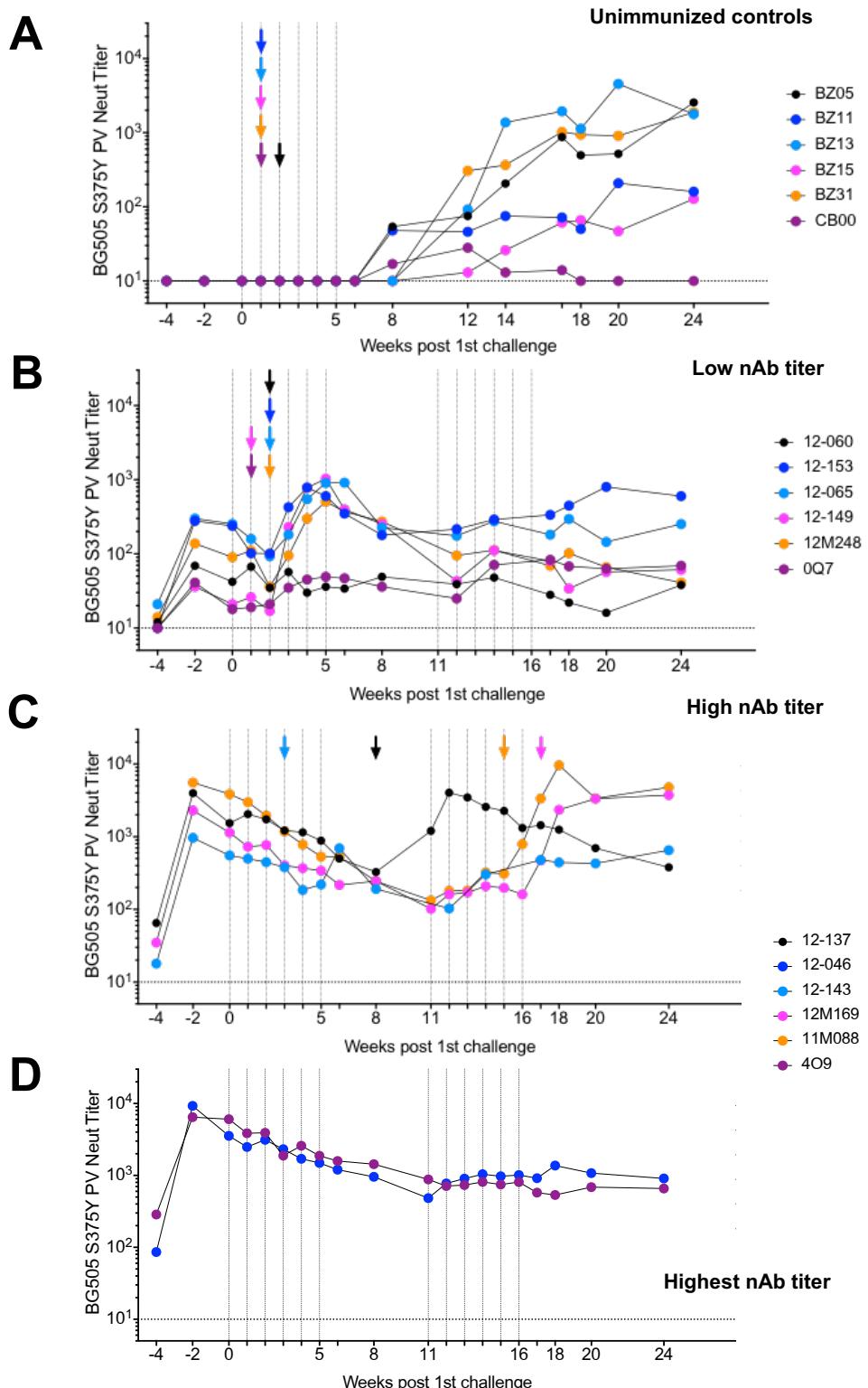
246 High nAb titer animals that became infected showed a comparable increase in  
247 BG505 S375Y nAb titers ~1-4 weeks following infection. The only exception was animal  
248 12-137, who suppressed viremia for 3 weeks following challenge at week 5, and thus  
249 delayed a surge in nAb titers until week 11 (Figure 3C). Animal 12-143, which became  
250 viremic at week 3, showed only a small rise in nAb titers at week 6, suggesting possible  
251 rapid viral escape. PacBio sequencing of viral species in 12-143 plasma at week 8 in fact  
252 revealed that >95% of sequenced *env* genomes contained putative escape mutations at  
253 residues 168 and 192 (Figure S3A). Similarly, *env* genomes in 12-137 plasma at weeks  
254 12 and 16 showed putative escape mutations at residues 354 and 356, flanking the N355  
255 glycan, which coincided with onset of nAb titer decay at week 12 (Figure S3B, Figure 3C).  
256 NAb specificities to the N355-region were observed in BG505 SOSIP immunized rabbits  
257 (Klasse et al., 2018), and were detected in week 0 plasma of animal 12-137 using electron

258 microscopy based serum mapping (Figure S3C) (Bianchi et al., 2018). We could further  
259 show that the observed viral point mutations do confer neutralization resistance to sera  
260 from the respective animals (Figure S3D-E). Animals 12M169 and 12M088, which  
261 become infected at weeks 16 and 14, respectively, exhibited slow declines in vaccine-  
262 induced nAb titers which then rose following infection (Figure 3C). The nAb titers of fully  
263 protected animals 12-046 and 4O9 (Figure 3D) initially declined and then plateaued at  
264 ~1:800 around week 10 and remained stable for the remainder of the study. This trend  
265 was mirrored in longitudinal ELISA EC<sub>50</sub> binding titers. (Figure S3F-G). Uninfected  
266 animals retained robust nAb titer levels over 1 year past the final immunization (Figure  
267 S3H).

268 The differences in both BG505 S375Y pseudovirus as well as SHIV<sub>BG505</sub> challenge  
269 stock neutralization ID<sub>50</sub> titers between high and low nAb titer animals at week -2 were,  
270 as anticipated, highly significant (Figure 1, B and C). Peak nAb titers at week -2 accurately  
271 predicted the duration of protection, identifying nAb titers as the primary correlate of  
272 protection ( $p < 0.0001$ , Figure 4A). Using the BG505 S375Y pseudovirus assay, a  
273 statistically significant difference was found between nAb titers in immunized animals 7d  
274 prior to onset of viremia and animals that remained uninfected until week 20 ( $p = 0.03$ ,  
275 Figure 4B, Figure S4A).

276 To numerically quantify the relationship between BG505 S375Y pseudovirus nAb  
277 titers and likelihood of infection, we developed a modified Bayesian logistic regression  
278 model using the neutralization and viral load data from all three animal groups (Figure  
279 4C, S5). The posterior median infection probability at the limit of nAb titer detection was  
280 77%, agreeing closely with an estimated animal infectious dose of 75% in unimmunized

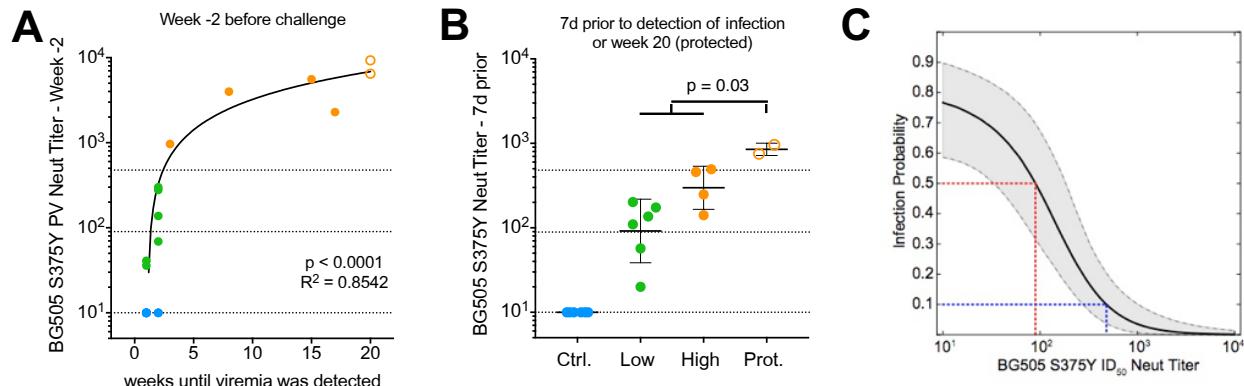
281 controls. A median per-challenge infection probability of 50% was attained with ID<sub>50</sub> titers  
282 of 1:90, which agrees well with the often-quoted 50% protective ID<sub>50</sub> titer of ~1:100,  
283 derived from bnAb passive transfer studies (Hessell et al., 2018; Moldt et al., 2012; Parren  
284 et al., 2001; Pegu et al., 2014; Shingai et al., 2014), although we note that various different  
285 neutralization assays with differing sensitivities were employed in these studies. To  
286 achieve an infection probability of 10%, or 90% protection, an ID<sub>50</sub> titer of 1:476 (CI: 272-  
287 991) was required. In agreement with our model, animals with nAb titers above ~1:500  
288 remained protected over all 12 challenges, while animals with nAb titers below 1:200  
289 generally became infected with only 1-2 challenges. For the rhesus CD4<sup>+</sup> T cell grown  
290 SHIV<sub>BG505</sub> S375Y virus stock, an ID<sub>50</sub> titer of ~1:30 (Figure S4B) was the protection  
291 threshold. The observed disparity between pseudovirus and PBMC-grown virus assays  
292 was relatively large compared with that reported for many mAbs but still within previously  
293 observed ranges (Figure S4C) (Cohen et al., 2018; Provine et al., 2012). Thus, Tier 2  
294 nAb titers both predicted and correlated with protection from infection.



295

296 **Figure 3: Longitudinal development of autologous Tier 2 nAb titers. (A-D) Serum**  
297 neutralizing antibody titers throughout the challenge schedule: BG505 S375Y

298 pseudovirus ID<sub>50</sub> nAb titers rise 8-12 weeks post infection in unimmunized animals (**A**) or  
299 1-2 weeks following detection of viremia in low nAb titer animals (**B**). BG505 S375Y  
300 pseudovirus ID<sub>50</sub> nAb titers in macaques that became infected over time (**C**) or showed  
301 sterilizing protection (**D**). In (**D**), nAb titers peaked at week -2 following a final boost at  
302 week -4 and slowly declined until ~week 10 after which titers plateaued and remained  
303 stable for the duration of the study. Macaques infected during the second set of  
304 challenges displayed similar nAb titer plateaus as protected animals until infection.  
305 Animals that became infected showed a surge in nAb titer followed by a slow decline e.g.  
306 animal 12-137. First detection of plasma viremia is indicated by colored arrows  
307 corresponding to the animal IDs shown in the respective figure legends.



308

309 **Figure 4. Protection is associated with serum nAb titers greater than ~1:500. (A)**  
310 BG505 S375Y pseudovirus ID<sub>50</sub> nAb titers at week -2 predict and correlate with the  
311 duration of protection. **(B)** BG505 S375Y pseudovirus ID<sub>50</sub> nAb titers of control (Ctrl.), low  
312 nAb titer (Low) and high nAb titer (High) animals 7 days before detection of viral load in  
313 the blood and at week 20 for protected (Prot.) animals that showed sterilizing protection  
314 throughout the study. All nAb titers were measured in TZM-bl assays. Correlations were  
315 calculated using Spearman correlation tests, comparisons between groups were  
316 calculated using Mann-Whitney U tests. Horizontal lines indicate 50% and 90% protective  
317 nAb titers as defined in 3G. **(C)** The 5%, median, and 95% credible intervals (CI) are  
318 shown for the probability of infection in relation to serum BG505 S375Y pseudovirus nAb  
319 titer, inferred using a modified Bayesian logistic regression model (see figs. S10-S12).  
320 The posterior median infection probability at the limit of nAb titer detection was 77%,  
321 agreeing closely with an estimated animal infectious dose of 75% in unimmunized  
322 animals. A median infection probability of 50% is attained with an ID<sub>50</sub> titer of 1:90 (red  
323 line, CI: 34-178), and an infection probability of 10% with an ID<sub>50</sub> titer of 1:476 (blue line,  
324 CI: 272-991).

325

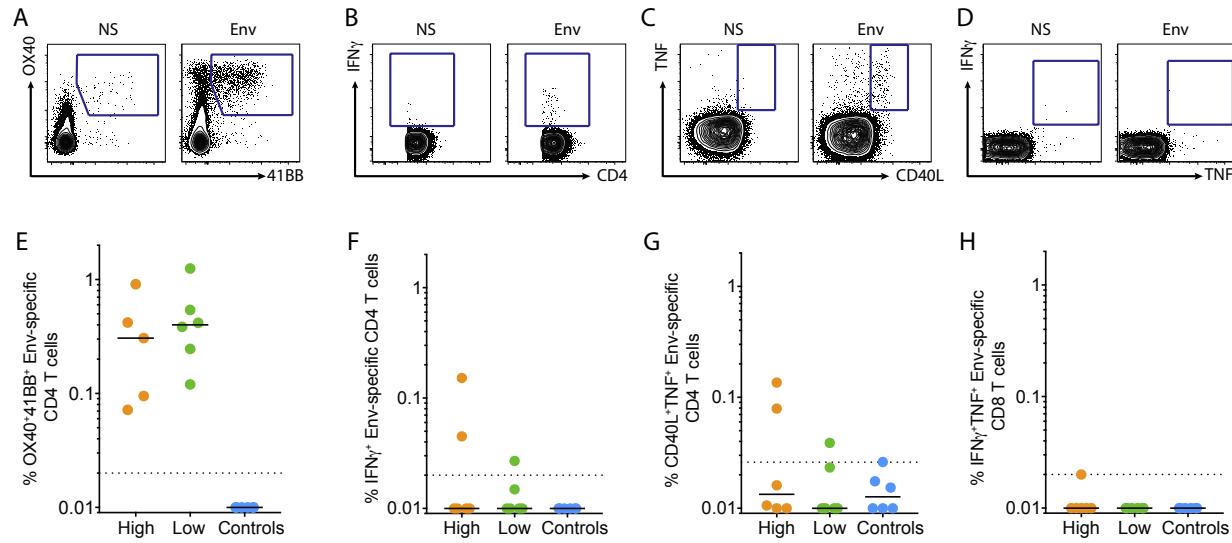
326 **T cell activity and serum antibody-dependent cell-mediated cytotoxicity (ADCC)**

327 **do not correlate with protection**

328 We further investigated other parameters that may have contributed to protection. Robust  
329 Env-specific CD4<sup>+</sup> T cell responses were elicited by BG505 Env trimer immunization and  
330 were equivalent in magnitude between the high and low nAb titer groups of immunized  
331 animals before challenge (Figure 5, A and E, Figure S6, A to C). Cytokine-producing Env-  
332 specific CD4<sup>+</sup> T cells were also comparable between the two groups of immunized  
333 animals before challenge (IFN $\gamma$ <sup>+</sup>, Figure 5, B and F; TNF<sup>+</sup>CD40L<sup>+</sup>, Figure 5, C and G,  
334 Figure S6D) and protein vaccine-elicited Env-specific CD8<sup>+</sup> T cells were undetectable, as  
335 expected (Figure 5,D and H). Thus, Env-specific CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells are not a  
336 correlate of protection.

337 Concerns have been raised about vaccine-elicited CD4<sup>+</sup> T cell responses  
338 enhancing susceptibility to infection by HIV (Fauci et al., 2014; Hu et al., 2014) or SIV  
339 (Fouts et al., 2015; Staprans et al., 2004) by providing more targets for infection at the  
340 mucosal site of transmission (Bukh et al., 2014; Carnathan et al., 2015; Martins and  
341 Watkins, 2017; Qureshi et al., 2012), most likely due to the presence of activated Th1  
342 cells in the mucosa, which was correlated with CCR5,  $\alpha$ 4 $\beta$ 7, or proliferation in different  
343 studies. Minimal Th1 cells were detected in the BG505 Env trimer immunized animals  
344 (IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells, Figure 5, B & F). CCR5<sup>+</sup>, Ki67<sup>+</sup> or Ki67<sup>+</sup>/ $\alpha$ 4 $\beta$ 7<sup>+</sup> CD4<sup>+</sup> T cells in  
345 peripheral blood prior to challenge were not correlated with susceptibility to infection or  
346 protection (Figure S6, E to H). Thus, we observed robust protection of high nAb titer  
347 animals against a mucosal SHIV challenge despite substantial levels of Env-specific  
348 vaccine-induced CD4<sup>+</sup> T cells in peripheral blood at 4 weeks after the final immunization.

349 The difference in our study may be due to a lack of Th1 or mucosal homing CD4+ T cells  
350 in response to the protein vaccine, compared to previously used viral vectors (Bukh et al.,  
351 2014; Carnathan et al., 2015; Fauci et al., 2014; Hu et al., 2014; Staprans et al., 2004).  
352 Alternatively, nAb-mediated protection against HIV/SIV may more readily overcome  
353 possible adverse consequences of increased numbers of activated CD4+ T cell targets  
354 than the non-neutralizing Abs (nnAbs) raised in the earlier studies.



355

356 **Figure 5. HIV Env-specific CD4<sup>+</sup> T cells and Env-specific CD8<sup>+</sup> T cells at week 0 are**  
357 **not associated with the observed protection from infection. (A-C)** Representative

358 flow plots of Env-specific CD4 T cells from week 0 PBMCs: using an OX40/4-1BB AIM

359 assay (Reiss et al., 2017) (A), ICS assay for IFN $\gamma$  (B), and ICS assay for TNF/CD40L (C)

360 when not stimulated (ns) versus stimulated with antigen (Env). (D) Representative flow

361 plot of IFN $\gamma$  and TNF expression in CD8 T cells by ICS when not stimulated (ns) versus

362 stimulated with antigen (Env). (E-G) Quantification of the percent of CD4 T cells that are

363 Env-specific based on: OX40/4-1BB (E), IFN $\gamma$  (F), or CD40L/TNF (G) expression. (H)

364 Quantification of the percent of CD8 T cells that are Env-specific based on IFN $\gamma$  and TNF

365 expression. Signal from the unstimulated condition was subtracted from the antigen-

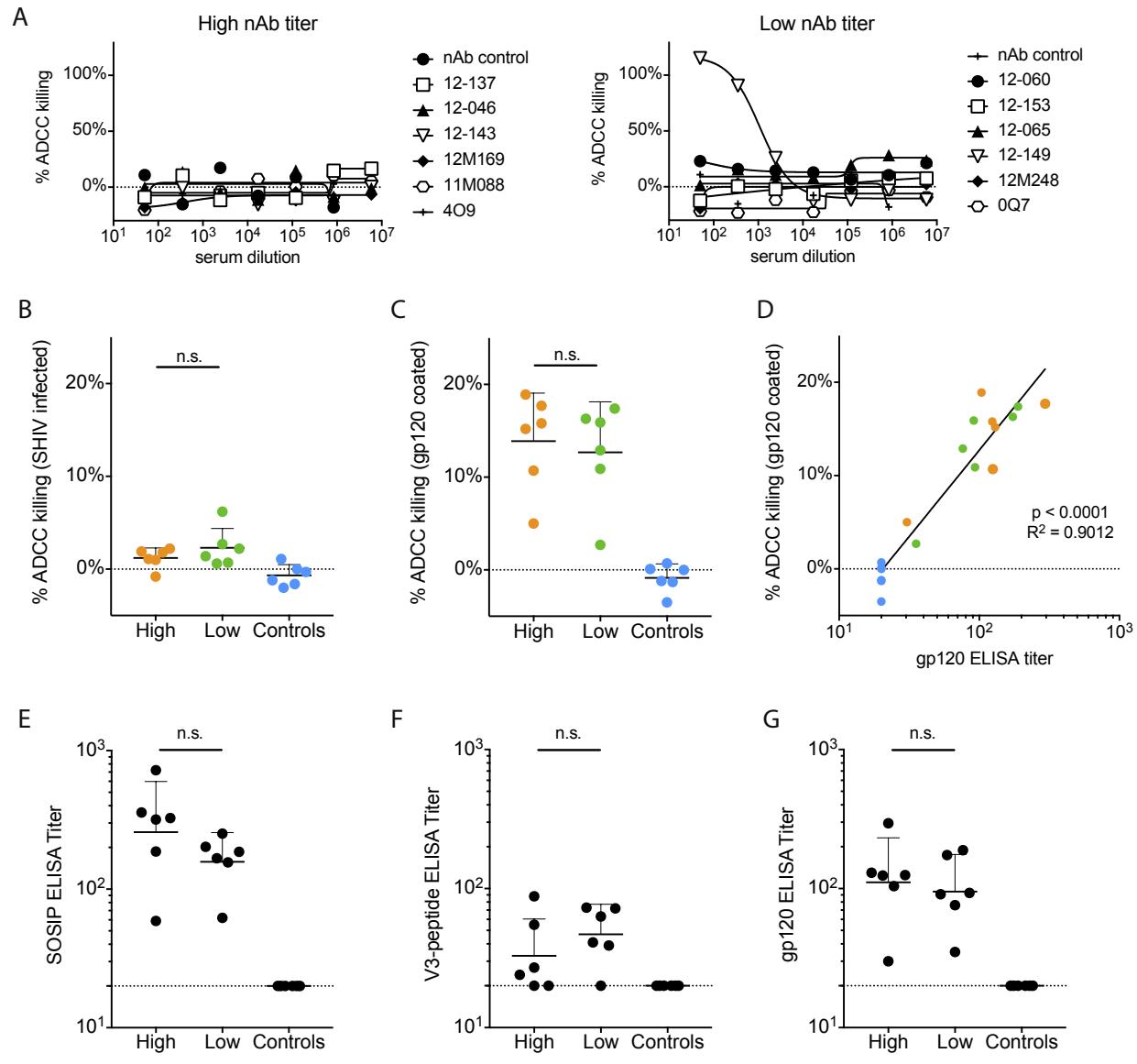
366 specific signal for each sample. Each dot represents an individual animal.

367 To investigate possible contributions of ADCC of both nAbs and nnAbs, we tested  
368 animal sera in two infection-based assays; SHIV<sub>BG505</sub>-infected CEM.NKR luciferase  
369 reporter cells (Alpert et al., 2012) (Figure 6A) and flow cytometric analysis of ADCC in  
370 p27<sup>+</sup> SHIV<sub>BG505</sub>-infected CEM.NKR target cells (Veillette et al., 2014) (Figure 6B). Using  
371 either assay, we failed to detect meaningful ADCC activity at week 0 with the exception  
372 of a single animal, 12-149, which was a low titer animal whose ADCC activity was non-  
373 specific and included activity against control SIV<sub>mac</sub>239 (Figure S7A). The absence of  
374 observed ADCC activity can be partially explained by the Tier 2 character of BG505 Env.  
375 In native Env trimer-based ADCC assays, nnAb and Tier 1 nAbs fail to mediate ADCC-  
376 activity against, hard-to-neutralize Tier 2 HIV isolates, as previously reported (Bredow et  
377 al., 2016; Ding et al., 2016). In addition, ADCC activation in infection-based assays varies  
378 strongly depending on the targeted epitope, which is likely related to the Ab binding  
379 stoichiometry to the epitope and the ability to cross-link sparse trimers on the virion  
380 surface (Figure S7B-D) (Bredow et al., 2016; Ding et al., 2016).

381 Unlike infection-based assays, ADCC killing measured on CEM.NKR target cells  
382 coated with BG505 gp120 was robust, but did not distinguish between high and low nAb  
383 titer animals and, therefore, was not associated with protection (Figure 6C). ADCC killing  
384 of gp120-coated cells did correlate with BG505 gp120 binding, indicating that gp120-  
385 binding antibodies are sufficient to induce ADCC in this assay (Figure 6D), but cannot  
386 mediate ADCC to native membrane-bound Env on infected cells. Thus, ADCC unlikely  
387 contributes to protection. We also observed considerable staining of p27<sup>-</sup> uninfected  
388 bystander T cells by both mAbs and animal sera, which appears to result from antibody  
389 binding to shed gp120 from infected cells that is captured on CD4 of uninfected cells

390 (Figure S7D) (Richard et al., 2018). Overall, these results suggest caution in the use of  
391 ADCC assays that are either based on recombinant gp120 or gp140 binding, rather than  
392 native Env on virus-infected cells, or cannot distinguish productively infected from  
393 uninfected bystander cells (Ackerman et al., 2016; Ferrari et al., 2011; Huang et al., 2016;  
394 Johansson et al., 2011; Kristensen et al., 2018). We note that the results pertain to ADCC;  
395 there remains the possibility that other Fc-mediated effector functions might contribute to  
396 protection.

397 Lastly, we determined BG505 SOSIP.664 (Figure 6E), V3-peptide (Figure 6F), and  
398 BG505 gp120 binding titers (Figure 6G) for all groups at week 0 since V3-targeting  
399 antibodies (Balasubramanian et al., 2018), and binding antibodies in general have been  
400 associated with anti-viral activities (Excler et al., 2014). No significant differences between  
401 high and low neutralizer animals were detected.



403 **Figure 6: ADCC activity at week 0 measured in SHIV-infection as well as gp120-  
404 based assays is not associated with the observed protection from infection. (A-G)**

405 ADCC-activity from sera of high and low nAb titer as well as control animals at week 0.

406 ADCC activity in titrated sera was measured using SHIV<sub>BG505</sub> challenge stock infected

407 CEM.NKR luciferase-reporter target cells and CD16 transfected KHYG-1 effector cells

408 (A) or in 1:250 diluted sera by flow cytometric analysis of ADCC activity in either p27<sup>+</sup>

409 SHIV<sub>BG505</sub>-infected CEM.NKR cells (B) or BG505 g120-coated CEM.NKR cells (C), using

410 PBMCs as effector cells. ADCC-activity in BG505 gp120-coated CEM.NKR cells  
411 correlated with BG505 gp120 binding titers (**D**). (**E-G**) ELISA EC<sub>50</sub> binding titers to: BG505  
412 SOSIP.664 (**E**), BG505 V3-peptide (**F**) or BG505 gp120 (**G**). Sera from high and low titer  
413 animals, as well as unimmunized control animals were tested for ELISA binding titers at  
414 week 0. Correlations were calculated using Spearman correlation tests, comparisons  
415 between groups were calculated using Mann-Whitney U tests.

416

417 **DISCUSSION**

418 Vaccine protection against HIV in humans and against SIV and SHIV in macaques has  
419 been associated with non-neutralizing antibodies (Barouch et al., 2015; Haynes et al.,  
420 2012). Here, we demonstrate that vaccine-induced Tier 2 nAbs, but not other antibody  
421 parameters such as V3 binding titers, antibody-dependent cellular cytotoxicity (ADCC),  
422 or induction of T cell activity, are a correlate of protection from homologous SHIV<sub>BG505</sub>  
423 infection in macaques. Notably, we employed a challenge dose of virus corresponding to  
424 an AID<sub>75</sub>, which sets a relatively high bar for protection, given that most animals (~53%)  
425 in the control arm were estimated to have been productively infected by two or more  
426 viruses (Table S2). Similar rates of multivariant virus transmission have been reported in  
427 men who have sex with men and injection drug users who acquire HIV-1 infection (38%  
428 and 60% with a MOI of 2 or higher, respectively), while heterosexual cohorts show lower  
429 multivariant transmission frequencies (~19%). Thus, our model mimics the conditions of  
430 productive transmission events, underlining the physiological relevance of the challenge  
431 dose that we used (Bar et al., 2010; Li et al., 2010).

432 We show, in the model system described, that animals remain protected from SHIV  
433 infection in a nAb titer-dependent manner, which suggests a strong relationship between  
434 circulating nAb titers in the blood and protection from mucosal challenge with difficult-to-  
435 neutralize, Tier 2 SHIV<sub>BG505</sub>. At the same time, our data suggest that vaccine protection  
436 can occur in the absence of ADCC. We show that unprotected animals have relatively  
437 high levels of ADCC when measured in a widely used ADCC assay that uses target cells  
438 coated with monomeric gp120, but not with SHIV<sub>BG505</sub> infected target cells. We further  
439 provide evidence that adjuvanted protein immunization with HIV Env can induce nAb titers

440 that are durable and protective over longer periods of time, if high initial nAb titers  
441 following immunization can be reached. This has been a major concern in the HIV vaccine  
442 field (Sundling et al., 2013), but also for other protein-based vaccines, such as  
443 recombinant influenza vaccines (Krammer and Palese, 2015). Importantly, we identified  
444 that a serum ID<sub>50</sub> nAb titer of ~1:500 against the homologous BG505 S375Y pseudovirus  
445 at the time of challenge can confer reliable protection of > 90%, meaning that 9 of 10  
446 challenges with a physiologically-relevant AID<sub>75</sub> dose would not result in infection. Finally,  
447 protection is observed for polyclonal neutralizing Ab responses that, as above and earlier  
448 {Pauthner:2017fd}, target multiple specificities on Env and not simply the previously  
449 described glycan hole on BG505 Env.

450 In conclusion, we provide evidence that protein immunization with native-like Env  
451 trimers can induce potent and protective nAb titers in the SHIV/macaque model. Thus,  
452 nAb-mediated protection from Tier 2 virus challenge is not limited to bnAbs, which are  
453 generally focused to a single site of vulnerability and have a defined effector-function  
454 profile, but can also be accomplished by polyclonal autologous nAb responses of  
455 sufficient magnitude and specificity, which comprise a broad range of neutralizing and  
456 non-neutralizing antibody lineages to various, often overlapping epitopes that interact in  
457 both synergistic or competitive ways (Klasse et al., 2018; Pauthner et al., 2017; Sanders  
458 et al., 2015; Torrents de la Peña et al., 2018). The protective nAb titer threshold against  
459 the homologous challenge virus that we determined is in rough accord with passive  
460 antibody transfer studies and provides a benchmark for comparison with upcoming  
461 antibody protection studies against HIV in humans ([www.ampstudy.org](http://www.ampstudy.org)).

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477 **AUTHOR CONTRIBUTIONS:**

478 The TSRI CHAVI-ID immunogen working group consisting of SC, WRS, ABW, IAW, RTW  
479 and DRB, with the assistance of MGP, STB, GMS and DHB designed the challenge study  
480 and laid out the experimental strategy. JPN and DHB oversaw all rhesus macaque  
481 immunizations and challenges, including sample acquisition, processing, storage and  
482 distribution. PA and DHB performed and oversaw viral load assays. HL and GMS  
483 designed and produced the SHIVBG505 challenge stock. CAC, DWK and TT with  
484 oversight from DJI, ABW, WRS and RWS designed and produced the boosting

485 immunogens for the study. MGP, RB, JHL and DRB designed HIV pseudovirus mutants  
486 and performed and oversaw neutralization experiments as well as ELISA binding  
487 experiments. CHD, SMR and SC performed and oversaw flow cytometric analysis of T  
488 cell activation. JP, RN and BVB with oversight from DTE, LH and AF performed ADCC  
489 assays. BN and MB with oversight from LH and ABW performed serum negative-stain  
490 EM analysis. BM and MGP performed statistical analysis of data sets. MGP, JPN, CHD,  
491 BM, SMR, JP, RN, BVB, TT, STB, DTE, LH, AF, IAW, RTW, DJI, WRS, ABW, RWS, SC,  
492 GMS, DHB and DRB analyzed data sets and contributed edits to the manuscript. MGP,  
493 SC and DRB wrote the manuscript.

494

#### 495 **DECLARATION OF INTERESTS**

496 The authors declare no competing interests

497

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860

861 **STAR METHODS**

862 **CONTACT FOR REAGENT AND RESOURCE SHARING**

863 Further information and requests for resources and reagents should be directed to and  
864 will be fulfilled by Dennis Burton ([burton@scripps.edu](mailto:burton@scripps.edu)).

865

866 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

867

868 **Rhesus macaques**

869 Outbred Indian rhesus macaques (*Macaca mulatta*) were sourced and housed at  
870 Alphagenesis Inc, Yemassee, SC and maintained in accordance with NIH guidelines.  
871 These studies were approved by the appropriate Institutional Animal Care and Use  
872 Committees (IACUC). None of the NHPs were previously enrolled in other studies that  
873 are not explicitly stated in the manuscript. All animals were genotyped for class I alleles  
874 Mamu-A\*01, Mamu-B\*08 and Mamu-B\*17 and Trim5, which are associated with  
875 spontaneous virological control. Genotype and gender information for all animals is  
876 reported in Table S1. Additional information on high- and low-nAb titer group animals is  
877 published in Pauthner et al., 2017.

878

879 **METHOD DETAILS**

880 **Rhesus monkey immunizations and challenge**

881 Animals were immunized at 4 weeks before challenge (week -4) with a fourth dose of the  
882 previously administered immunogen for a given animal with adjuvant (Figure S1B)

883 (Pauthner et al., 2017). The adjuvant used for this boost was an ISCOMATRIX-like  
884 nanoparticle comprised of self-assembled cholesterol, phospholipid, and Quillaja saponin  
885 prepared as previously described (Lovgren-Bengtsson et al., n.d.). All immunizations  
886 were administered as split doses. Each immunization consisted of two subcutaneous  
887 injections of 50 µg of Env trimer protein + 187.5 units (U) of saponin adjuvant, in sterile  
888 phosphate-buffered saline (PBS) diluent for a total of 100 µg of Env trimer protein + 375  
889 U of Iscomatrix per immunization per animal. Subcutaneous immunizations were given in  
890 a volume of 0.5 ml with a 1 inch, 25-gauge needle at the medial inner mid-thigh of each  
891 leg. The subcutaneous injection technique consists of making a 'skin tent' and inserting  
892 the needle into the subcutaneous space at a 45° angle.

893 Serum was collected in SST Vacutainer tubes (BD Biosciences) and processed  
894 according to the manufacturer's instructions. Multiple aliquots of 0.5 ml were frozen at  
895 -80° C. Whole blood was collected in K2 EDTA Vacutainer tubes (BD Biosciences) for  
896 plasma and PBMC isolation. Multiple aliquots of 0.5 ml of plasma were frozen at -80° C.  
897 PBMCs were isolated using Thermo Scientific Nunc EZFlip Conical Centrifuge Tubes per  
898 manufacturer's instructions. PBMCs were isolated, counted, and re-suspended at  $1 \times 10^7$   
899 cells/mL in FBS containing 10% DMSO. Aliquots were subsequently frozen in 1 mL vials  
900 using a Mr. Frosty freezing container (Nalgene, cooling rate of 1°C / minute) and placed  
901 in a -80° C freezer. The following day PBMC samples were moved to storage in a liquid  
902 nitrogen freezer tank.

903 Animals were atraumatically inoculated intrarectally with a 1:75 dilution of rhCD4-  
904 grown SHIVBG505 N332 S375Y  $\Delta$ CT challenge stock (Li et al., 2016) in RPMI 1640  
905 (Gibco), which amounted to  $1.4 \times 10^7$  virions or 2 ng p27. See dataset S1B in Li et al. (Li

906 et al., 2016) for a complete characterization of the challenge stock with respect to virion  
907 content and virion infectivity.

908

909 **Viral Load Assay**

910 Plasma SHIV RNA levels in serum following infection were measured using a *gag*-  
911 targeted quantitative real-time RT-PCR assay as previously described (Hansen et al.,  
912 2013).

913

914 **Serum neutralization assays**

915 Replication incompetent HIV pseudovirus was produced by co-transfected *env* plasmids  
916 with an *env*-deficient backbone plasmid (pSG3Δ*env*) in HEK293T cells in a 1:2 ratio, using  
917 the X-tremeGENE 9 transfection reagent (Roche). Pseudovirus was harvested after 48-  
918 72 h by sterile-filtration (0.22 µm) of cell culture supernatants, and neutralization was  
919 tested by incubating pseudovirus and serum or mAbs for 1 h at 37 °C before transferring  
920 them onto TZM-bl cells as previously described (Pauthner et al., 2017). For replication  
921 competent SHIV<sub>BG505</sub> neutralization, rhCD4-grown SHIV<sub>BG505</sub> N332 S375Y challenge  
922 stock was used instead in a BSL3 facility with no further modifications.

923 Neutralization is measured in duplicate wells within each experiment. BG505 nAb  
924 titers for group comparisons were measured in three or more independent experiments  
925 that were subsequently averaged. The BG505 pseudovirus time course neutralization  
926 data shown in Figure 3 were generated in single large experiments, to test sera from all  
927 time points side-by-side, thus ensuring the highest nAb titer comparability between time

928 points. Neutralization was tested starting at 1:10 serum dilutions followed by nine serial  
929 3-fold dilutions to ensure the highest sensitivity and range of detection. Neutralization ID<sub>50</sub>  
930 titers were calculated using the 'One site – Fit logIC<sub>50</sub>' regression in Graphpad Prism v7.0.  
931 ID<sub>50</sub> nAb titers of incomplete neutralization curves that reached at least 50%, but less than  
932 90% maximal neutralization, were calculated by constraining the regression fit through  
933 0% and 100% neutralization, to ensure accurate calculation of half-way (50%) nAb titers.  
934 All neutralization titers are reported as ID<sub>50</sub> titers. All nAb titer data panels show geometric  
935 mean titers with geometric SD. BG505 pseudovirus neutralization was tested using the  
936 BG505.W6M.ENV.C2 isolate (AIDS Reagents Program), carrying the T332N mutation to  
937 restore the N332 glycosylation site, as well as other indicated mutations that were added  
938 by site-directed mutagenesis.

939

#### 940 **Serum binding ELISAs**

941 Microlon 96-well plates (Corning) were coated overnight with streptavidin at 2.5 µg/mL  
942 (Thermo Scientific). Plates were then washed 4-5 times with PBS-tween (0.05%) and  
943 blocked with PBS + 3% BSA for 1 h at room temperature. If capturing biotinylated BG505  
944 SOSIP.664-Avi or BG505-Avi gp120, proteins were added at 2.5 µg/mL in PBS + 1% BSA  
945 for 2 h at room temperature. For V3-peptide binding assays, no streptavidin was coated  
946 and instead BG505 V3-peptide (TRPNNNTRKSIRIGPGQAFYATG) was directly coated  
947 to Microlon 96-well plates at 2.5 µg/mL in PBS overnight. Plates were then washed 4-5  
948 times with PBS-tween (0.05%) and serially diluted sera in PBS + 1% BSA were then  
949 added for 1 h at room temperature. Plates were then washed 4-5 times with PBS-tween  
950 (0.05%) and alkaline phosphatase-conjugated goat anti-human IgG (Jackson

951 ImmunoResearch) was added for 1 h at a 1:1000 dilution (final concentration 0.33 µg/mL)  
952 in PBS + 1% BSA at room temperature. Plates were then washed 4-5 times with PBS-  
953 tween (0.05%) and absorption at 405 nm was measured following addition of  
954 phosphatase substrate in alkaline phosphatase buffer. We calculated half maximal EC<sub>50</sub>  
955 binding titers using Graphpad Prism v7.0. All ELISA Ab data panels show geometric mean  
956 titers with geometric SD.

957

## 958 **ADCC assays**

959 *Luciferase-based CEM.NKR SHIV, HIV, SIV infection assay*

960 ADCC activity was measured as previously described (Alpert et al., 2012). CEM.NKR-  
961 CCR5-sLTR-Luc cells, which express luciferase (Luc) upon infection, were infected with  
962 either HIV-1 BG505, SHIV BG505 or SIV<sub>mac</sub>239 by spinoculation in the presence of 40  
963 µg/ml of polybrene. For HIV-1 BG505 and SHIV<sub>BG505</sub> infections, *vif*-deleted infectious  
964 molecular clones were pseudotyped with Vesicular stomatitis virus G (VSVG). Two days  
965 post-infection with VSVG-pseudotyped HIV-1/SHIV<sub>BG505</sub> and 4 days post-infection with  
966 SIV<sub>mac</sub>239, CEM.NKR-CCR5-sLTR-Luc cells were incubated at a 10:1 effector:target cell  
967 ratio either with an NK cell line expressing rhesus macaque CD16 in the presence of  
968 serial dilutions of rhesus macaque sera or an NK cell line expressing human CD16 in the  
969 presence of human monoclonal bnAbs. After an 8-hour incubation, Luc activity was  
970 measured using BriteLite luciferase substrate (PerkinElmer). Uninfected or infected cells  
971 incubated with NK cells in the absence of antibody or plasma were used to determine  
972 background and maximal Luc activity, respectively. The dose-dependent loss of Luc  
973 activity represents the antibody-dependent killing of productively infected target cells.

974

975 *FACS-based CEM.NKR SHIV infection assay*

976 VSVG-pseudotyped SHIV<sub>BG505</sub> N332 S375Y virus was produced and titrated as  
977 previously described (Veillette et al., 2015). Viruses were then used to infect CEM.NKR-  
978 CCR5-sLTR-Luc cells by spin infection at 800 × g for 1 h in 96-well plates at 25 °C.  
979 Measurement of ADCC using the FACS-based assay was performed at 48h post-infection  
980 as previously described (Veillette et al., 2015). Briefly, infected CEM.NKR-CCR5-sLTR-  
981 Luc cells were stained with viability (AquaVivid; Thermo Fisher Scientific) and cellular (cell  
982 proliferation dye eFluor670; eBioscience) markers and used as target cells. Human  
983 PBMCs isolated from three different healthy HIV-uninfected individuals were used as  
984 effector cells and were stained with another cellular marker (cell proliferation dye  
985 eFluor450; eBioscience). Effector cells were added at an effector:target cell ratio of 10:1  
986 in 96-well V-bottom plates (Corning, Corning, NY). A 1:250 final dilution of sera or 5 µg/ml  
987 of mAbs were added to appropriate wells and cells were incubated for 15 min at room  
988 temperature. The plates were subsequently centrifuged for 1 min at 300 g, and incubated  
989 at 37°C, 5% CO<sub>2</sub> for 5 to 6 h before being fixed with a PBS-formaldehyde solution (2%  
990 formaldehyde final concentration). Cells were then permeabilized using the  
991 Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and SHIV-infected cells  
992 were identified by intracellular staining using Alexa fluor 488-conjugated anti-p27 Abs  
993 (clone 2F12). Samples were analyzed on an LSRII cytometer (BD Biosciences). Data  
994 analysis was performed using FlowJo vX.0.7 (Tree Star). The percentage of ADCC was  
995 calculated with the following formula: (% of p27+ cells in Targets plus Effectors) – (% of  
996 p27+ cells in Targets plus Effectors plus Abs or sera) / (% of p27+ cells in Targets) by

997 gating on infected living target cells. Of note, samples were deidentified and tested and  
998 analyzed blindly.

999

1000 *FACS-based gp120-coated CEM.NKR ADCC assay*

1001 CEM.NKR-CCR5-sLTR-Luc cells were coated with 1 $\mu$ g of recombinant HIV-1<sub>BG505</sub> N332  
1002 gp120/million cells for 30 min at 37°C. gp120-coated target cells were used as target cells  
1003 and were stained with viability (AquaVivid; Thermo Fisher Scientific) and cellular (cell  
1004 proliferation dye eFluor670; eBioscience) markers. ADCC was performed as described  
1005 above with the difference that after target/effector cells co-incubation, cells were fixed  
1006 with a PBS-formaldehyde solution (2% formaldehyde final concentration) containing a  
1007 constant number of flow cytometry particles (5 $\times$ 10<sup>4</sup>/ml) (AccuCount Blank Particles, 5.3  
1008  $\mu$ m; Spherotech, Lake Forest, IL, USA). These particles are designed to be used as  
1009 reference particles since their concentration is known, thus allowing to count the absolute  
1010 cell number by flow cytometry. A constant number of particles (1 $\times$ 10<sup>3</sup>) were counted  
1011 during cytometry acquisition in order to normalize the number of viable targets cells. Each  
1012 sample was acquired with a LSRII (BD Bioscience, Mississauga, ON, Canada) and data  
1013 analysis was performed using FlowJo vX.0.6 (Tree Star, Ashland, OR, USA). The  
1014 percentage of ADCC was calculated with the following formula: (relative count of gp120-  
1015 coated cells in targets plus effectors) - (relative count of gp120-coated cells in targets plus  
1016 effectors plus Abs or sera) / (relative count of gp120-coated cells in targets) by gating live  
1017 target cells (Veillette et al., 2015). Of note, samples were deidentified and tested and  
1018 analyzed blindly.

1019

1020 **T cell analysis**

1021 Frozen aliquots of macaque PBMCs were thawed, washed once with RPMI + 10% FBS  
1022 (R10), incubated with DNase (100ug/ml, StemCell Technologies 07900) for 15 minutes  
1023 at 37C, then washed again and split in half for a CD8<sup>+</sup> ICS assay and a CD4<sup>+</sup> T cell  
1024 Activation Induced Marker (AIM) assay (Reiss et al., 2017).

1025

1026 For the CD8+ T cell ICS assay, the sample was further split into three groups and either  
1027 left unstimulated (NS), stimulated with BG505 Env peptides (5ug/ml), or stimulated with  
1028 SEB (1ug/ml) for 2 hours at 37C. Brefeldin A was then added (2ug/ml), and the  
1029 stimulations incubated for another 4 hours at 37C. The cells were then stained for 30  
1030 minutes at 4C with the fluorescent antibodies in the Surface Marker Panel below and  
1031 washed twice with FACS buffer. They were fixed with eBio intranuclear fix/perm kit for 20  
1032 minutes, washed once with perm buffer, then stained with the antibodies in the  
1033 Intranuclear Panel in perm buffer for 30 minutes at 4C. The samples were then washed  
1034 with FACS buffer and acquired on a BD LSR Fortessa.

1035

1036 For the CD4<sup>+</sup> T cell AIM assay, the sample was further split into three groups and either  
1037 left unstimulated (NS) or stimulated with BG505 Env peptides (5ug/ml), or stimulated with  
1038 SEB (100 pg/ml) for 24 hours at 37C. The cells were then stained for 60 minutes at 4C  
1039 with the fluorescent antibodies in the AIM Surface Marker Panel below, washed with  
1040 FACS buffer, fixed with 1% formaldehyde for 10 minutes at 4C, then washed again before  
1041 acquisition on a BD LSR Fortessa.

1042	CD8 Surface Marker Panel:		
1043	CD4 (Clone SK3)	PerCP	1:200
1044	CD20 (Clone 2H7)	BV570	1:200
1045	CD8 (Clone RPA-T8)	BV650	1:200
1046	CCR5 (3A9)	APC	1:200
1047	a4b7 (Act-1)	PE	1:200
1048	Live/Dead	APC e780	1:1000
1049			
1050	CD8 Intranuclear Panel:		
1051	Ki67 (B56)	Ax488	1:100
1052	IL2 (MQ1-17H12)	Ax700	1:100
1053	IFN (Clone B27)	Pac Blue	1:100
1054	TNF (MAb11)	PECy7	1:100
1055	CD40L (24-31)	BV605	1:100
1056			
1057	CD4 T Cell AIM Surface Marker Panel:		
1058	CD4 (Clone OKT-4)	BV650	1:100
1059	CD20 (Clone 2H7)	BV570	1:100
1060	PD1 (Clone EH12.2H7)	BV785	1:100
1061	CXCR5 (Clone MU5UBEE)	PECy7	1:100
1062	Live/Dead	APC e780	1:1000
1063	CD14 (61D3)	APC e780	1:100
1064	CD16 (eBioCD16)	APC e780	1:100
1065	CD25 (Clone BC96)	FITC	1:100
1066	Ox40 (Clone L106)	PE	1:100
1067	4-1-BB (4B4-1)	BV421	1:100
1068	ICOS (C398.4A)	PerCP Cy5.5	1:100
1069	CXCR3 (1C6)	APC	1:100
1070			

1071 **Full length env viral sequencing**

1072 *Long-read env sequencing*

1073 Samples were processed using the full-length Env sequencing protocol developed in  
1074 (Laird Smith et al., 2016), but with modified primers and PCR conditions. Briefly, plasma  
1075 samples were pelleted through a sucrose cushion to enrich for virions, RNA was extracted  
1076 using the QIAamp Viral RNA Mini Kit (part no. 52906; Qiagen, Valencia, CA), and cDNA  
1077 generated using the SuperScript III First Strand Synthesis System for RT-PCR (part no.  
1078 18080-051; Thermo Fisher, Fremont, CA), with oligo (dT) primers. SHIV env was  
1079 amplified from this cDNA using the HIV env forward primer from (Laird Smith et al., 2016)  
1080 Env-F: GAGCAGAAGACAGTGGCAATGA, and using a reverse primer designed for this  
1081 SHIV: CCCTGATTGTATTCTGTCCCTCAC, both purchased (de-salted) from Integrated  
1082 DNA Technologies (San Diego, CA) and diluted to 20 pmol in 0.1X TE buffer before use.  
1083 PCR was as in (Laird Smith et al., 2016), using the Advantage 2 PCR reaction mixture  
1084 (Advantage 2 PCR Kit, catalog no. 639206; Clontech, Mountain View, CA), with the SA  
1085 Buffer, but using 42 cycles of 15 sec denaturation at 95°C, 30 sec annealing at 64°C, and  
1086 3 min extension at 68°C. A QIAquick PCR Purification Kit (part no. 28106; Qiagen,  
1087 Valencia, CA) was used to purify PCR products, and Pacific Biosciences library  
1088 preparation was exactly as in (Laird Smith et al., 2016), but using the newer P6/C4  
1089 chemistry, and with a modified 0.025nM loading concentration, and a 6 hour movie time.  
1090 The challenge stock was handled identically but was highly concentrated and thus only  
1091 23 PCR cycles were used during amplification.

1092

1093 *PacBio env data processing*

1094 An updated version of the Full-Length Env Analyzer (Eren et al., 2017; Laird Smith et al.,  
1095 2016) pipeline was used to process SIV PacBio reads. Briefly, PacBio's CCS2 algorithm  
1096 was used to reconstruct single molecule Circular Consensus Sequence (CCS) reads,  
1097 outputting fastq files. These reads were filtered for length, quality, and for matching an  
1098 Env reference database (here we included the known BG505.SHIV challenge sequence)  
1099 with FLEA's default parameter settings. FLEA's error correction and data-summarizing  
1100 approach was used, again with default parameters, collapsing near-identical reads and  
1101 generating high-quality consensus sequences (HQCSs), along with HQCS frequencies,  
1102 which are then codon aligned. These HQCS sequences are visualized in a web browser  
1103 environment, allowing the exploration of immunotype frequencies, and displaying variants  
1104 upon the leaf nodes of a maximum likelihood phylogeny. Variant frequencies in Figure  
1105 S3A-B were computed from HQCS sequence frequencies.

1106

### 1107 **Complex preparation for negative-stain EM**

1108 Serum Fab preparation was carried out as previously described (Bianchi et al., 2018). In  
1109 brief, after buffer exchanging into TBS, up to ~1 mg of total Fab was incubated overnight  
1110 with 10-15 µg BG505 trimers at RT in ~50 µL total volume. Complexes were then purified  
1111 via size exclusion chromatography (SEC) using Superose 6 Increase 10/300 column (GE  
1112 Healthcare) in order to remove unbound Fab. The flow-through fractions containing the  
1113 complexes were pooled and concentrated using 100 kDa cutoff centrifugal filters (EMD  
1114 Millipore). The final trimer concentration was titrated to 0.04 mg/mL prior to application  
1115 onto carbon-coated copper grids.

1116

1117 **Negative-stain EM**

1118 The SEC-purified complexes were applied to glow-discharged, carbon-coated 400-mesh  
1119 copper grids, followed by pipetting 3  $\mu$ l of 2% (w/v) uranyl formate stain and blotting,  
1120 followed by application of another 3  $\mu$ l of stain for 45–60 s, again followed by blotting.  
1121 Stained grids were stored under ambient conditions until ready for imaging. Images were  
1122 collected via Leginon software using a Tecnai T12 electron microscopes operated at 120  
1123 kV  $\times$ 52,000 magnification. In all cases, the electron dose was 25 e $^-$ /A $^2$ . Particles were  
1124 picked from the raw images using DoG Picker and placed into stacks using Appion  
1125 software. 2D reference-free alignment was performed using iterative MSA/MRA. The  
1126 particle stacks were then converted from IMAGIC to RELION-formatted MRC stacks and  
1127 subjected to RELION 2.1 2D and 3D classification. A detailed protocol can be found in  
1128 Bianchi et al., Immunity 2018.

1129

1130 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1131

1132 Infection probability per challenge event was modeled as depending on the BG505 N332  
1133 S375Y log<sub>10</sub> ID<sub>50</sub> nAb titer at the time of challenge using a modified logistic regression,  
1134 where the maximum infection probability (where 0 < max < 1) was an additional parameter  
1135 to be estimated by the model, rather than being fixed at 1 as in traditional logistic  
1136 regression:

$$\frac{\text{max}}{1 + e^{-\text{slope}(\text{x}-\text{offset})}}$$

1137

1138 This adjustment is necessary because unimmunized animals with no serum nAb titers  
1139 are not infected with 100% probability upon the first challenge, as a consequence of the  
1140 chosen AID<sub>75</sub> challenge dose. The infection event was assumed to be the challenge time  
1141 point prior to the detection of viremia. Per-time point challenge outcomes were assumed  
1142 to be conditionally independent of each other when conditioning on the corresponding  
1143 BG505 N332 S375Y log10 ID<sub>50</sub> nAb titer of the respective time point. We assumed weakly  
1144 informative priors over the three model parameters, with slope~*Normal*(0,10),  
1145 offset~*Normal*(0,10), and max~*Uniform*(0,1), and we used the Metropolis algorithm to  
1146 draw 1 million samples from the posterior distribution. Chain mixing was rapid (see trace  
1147 plots in Figure S5B), with effective sample sizes (ESSs) above 20,000 for all 3 parameters  
1148 and for the log posterior probability. The posterior parameter distributions are visualized  
1149 in Figure S5A. The calculated 5%, 50%, and 95% quantiles for each parameter were:

1150

1151 slope: -6.32937, -3.49356, -1.99455

1152 offset: 1.71374, 2.12005, 2.4339

1153 max: 0.602868, 0.80477, 0.962382

1154

1155 While under the prior distribution,  $P(\text{slope} < 0) = 0.5$  and  $P(\text{slope} > 0) = 0.5$ , allowing  
1156 equal prior probability of protective or sensitizing effects of neutralizing antibodies, the  
1157 posterior probability of  $P(\text{slope} < 0) = 1$  indicated the strongest possible evidence for  
1158 decreasing infection probabilities given increasing ID<sub>50</sub> nAb titers. Figure S5C shows

1159 10,000 posterior sampled logistic curves, and the 5%, median, and 95% credible intervals  
1160 for the infection probability computed from these, that were used to plot Figure 4C.

1161

1162 Graphpad Prism v7.0 was used for all standard statistical analyses. The significance of  
1163 differences in neutralization and binding data between groups was calculated using  
1164 unpaired, two-tailed Mann-Whitney U tests, correlations were calculated using Spearman  
1165 correlation tests. Statistical parameters of all analyses are reported in the respective  
1166 figure legends.

1167

1168 **KEY RESOURCES TABLE**