

HIV

Partial efficacy of a broadly neutralizing antibody against cell-associated SHIV infection

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Broadly neutralizing antibodies (BnAbs) protect macaques from cell-free simian/human immunodeficiency virus (SHIV) challenge, but their efficacy against cell-associated SHIV is unclear. Virus in cell-associated format is highly infectious, present in transmission-competent bodily fluids, and potentially capable of evading antibody-mediated neutralization. The PGT121 BnAb, which recognizes an epitope consisting of the V3 loop and envelope glycans, mediates antibody-dependent cellular cytotoxicity and neutralization of cell-to-cell HIV-1 transmission. To evaluate whether a BnAb can prevent infection after cell-associated viral challenge, we infused pigtail macaques with PGT121 or an isotype control and challenged animals 1 hour later intravenously with SHIV_{SF162P3}-infected splenocytes. All five controls had high viremia 1 week after challenge. Three of six PGT121-infused animals were completely protected, two of six animals had a 1-week delay in onset of high viremia, and one animal had a 7-week delay in onset of viremia. The infused antibody had decayed on average to 2.0 µg/ml by 1 week after infusion and was well below 1 µg/ml (range, <0.1 to 0.8 µg/ml) by 8 weeks. The animals with a 1-week delay before high viremia had relatively lower plasma concentrations of PGT121. Transfer of 22 million peripheral blood mononuclear cells (PBMCs) stored at weeks 1 to 4 from the animal with the 7-week delayed onset of viremia into uninfected macaques did not initiate infection. Our results show that HIV-1-specific neutralizing antibodies have partial efficacy against cell-associated virus exposure in macaques. We conclude that sustaining high concentrations of bioavailable BnAb is important for protecting against cell-associated virus.

INTRODUCTION

The generation of broadly neutralizing antibodies (BnAbs) capable of potentially inhibiting diverse HIV-1 strains is widely viewed as an ideal property of an HIV-1 vaccine. Administration of several BnAbs or BnAb-like molecules intravenously, mucosally, or via viral vectors protects against intravenous and/or mucosal cell-free simian/human immunodeficiency virus (SHIV) challenge in macaques and HIV-1 challenges in humanized mice (1–8). Studies of BnAbs with mutated Fc domains suggest that Fc-dependent antibody functions, but not complement functions, can contribute to the protection conferred against cell-free SHIV by BnAbs (2). Given the prophylactic potential of BnAbs, there are major efforts under way to induce BnAbs by vaccination or to supply BnAbs exogenously using passive administration or gene-mediated transfer (1, 9).

Although most studies of HIV-1 transmission are conducted using cell-free virus, it has long been hypothesized that cell-associated HIV-1 (so-called “Trojan horse HIV”) could account for a proportion of newly acquired infections (10–12). Semen and other infectious body fluids, such as breast milk, contain both cell-free and cell-associated HIV-1 (13, 14). Studies in macaques demonstrate that cell-associated simian

immunodeficiency virus (SIV) can readily initiate infections (15–17). A small study comparing viral genetic signatures of cell-free and cell-associated virus in seminal fluid from the transmitting partner to the founder virus within the newly infected partner suggested that cell-associated virus might initiate at least a proportion (that is, three of five) of new human HIV-1 infections (14). A major implication of cell-to-cell transmission of HIV-1 is that the mechanism may allow the virus to evade several BnAbs (18–20). Evidence suggests that steric hindrance might reduce BnAb efficacy for blockade of virus transmission across virological synapses, where transmission of virus from one cell to another occurs (21). Furthermore, concerns exist about the potency of some gp120-specific BnAbs against envelope conformations taken by immature virions on acutely infected cells. Some BnAbs, such as PGT121, which recognizes an epitope consisting of the V3 loop and envelope glycans (22), might have the potential to efficiently block in vivo cell-associated virus challenges. Malbec *et al.* (23) demonstrated that PGT121 efficiently neutralized cell-associated HIV-1 in vitro through accessing the virological synapse. Note that a subsequent study by Li *et al.* (24), which used a different in vitro readout, showed PGT121 to have a lower in vitro maximum neutralization of cell-associated than cell-free HIV-1. In addition to neutralizing viral transmission, BnAbs may potentially confer protection from cell-associated virus through eliminating the infected cells via antibody-dependent cellular cytotoxicity (ADCC). BnAbs have recently been shown to efficiently eliminate HIV-1-infected cells in a murine model of HIV-1 infection (25). The ability to mediate ADCC has been demonstrated for PGT121 (26).

Although some BnAbs can efficiently neutralize cell-associated virus in vitro, the degree to which BnAbs can prevent in vivo infections initiated with cell-associated virus is not known. Virus within infected cells may transmit to host cells through a cell-to-cell transmission process that is relatively hidden from BnAb responses. The infection of even a

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limited number of host cells may allow the virus to lie dormant for a period of time until BnAb immunity fades. Preexposure prophylactic treatment models with long-acting antiretrovirals in both mice and nonhuman primates have revealed establishment of cryptic infections that remain undetectable until drug concentrations wane (27, 28). We assessed whether passive administration of the PGT121 BnAb could prevent infection of pigtail macaques after challenge with high-dose cell-associated SHIV_{SF162P3}.

RESULTS

Cell-associated SHIV_{SF162P3} infection of macaques

We first established an intravenous cell-associated SHIV infection model in macaques using primary allogeneic cells. One pigtail macaque was infected intravenously with SHIV_{SF162P3} and euthanized 2 weeks after infection when plasma viremia was high at 6.6 log₁₀ copies/ml (Fig. 1A). This animal's spleen was harvested, and a suspension of splenic mononuclear cells was prepared and viably cryopreserved. We proceeded to use intravenous administration of 2.45×10^7 splenocytes per intravenous challenge. Initially, one control animal, which was not given isotype control antibody, was challenged with the cell-associated virus stock. This animal exhibited high viremia within 1 week of exposure (Fig. 1B, dotted line). An additional four control animals, which were administered an isotype control antibody (1 mg/kg) 1 hour before challenge, exhibited similarly high viremia early after challenge (Fig. 1B, solid lines). The viremia during the first 4 weeks of infection after this cell-associated SHIV_{SF162P3} challenge was not significantly different to previous studies of mucosal cell-free SHIV_{SF162P3} infection in

pigtailed macaques using the same stock [time-weighted average plasma SHIV RNA viral load (TWA VL) difference = 0.27 log₁₀ copies/ml, $P = 0.52$; fig. S1A] (29). As expected with SHIV_{SF162P3} (30), a proportion (that is, two of five) of infected animals eventually controlled viremia, whereas two of the animals with ongoing viremia experienced a CD4 T cell decline and mild weight loss during follow-up and were euthanized. A limited in vivo titration showed that the amount of this cell-associated SHIV stock used had an animal infectious dose of ~1000 because dilutions of the stock were infectious in single animals at 1:100 and 1:1000 dilutions but not at 1:10,000 or 1:100,000 dilutions (fig. S2).

We investigated whether the BnAb PGT121 could protect animals from an intravenous cell-associated SHIV_{SF162P3} challenge. We chose to study PGT121 because this BnAb showed an ability to limit cell-to-cell HIV transmission in vitro and an ability to mediate ADCC, which may be important in clearing infected cells (23, 26). We confirmed that PGT121 was able to bind pigtail macaque Fcγ receptors (FcγRs) and mediate Env-specific ADCC of HIV-1-infected cells using pigtail macaque effector cells (figs. S3 and S4).

The test animals received PGT121 [1 mg/kg, intravenously (iv)] 1 hour before challenge with 2.45×10^7 splenocytes from the SHIV_{SF162P3}-infected donor. The dose (1 mg/kg) was chosen because it had previously completely protected rhesus macaques from a mucosal cell-free SHIV_{SF162P3} challenge (3). Three of the six pigtail macaques receiving PGT121 before challenge exhibited no detectable viremia at any time point after challenge (Fig. 1C, solid lines), whereas the other three PGT121-infused macaques exhibited a delay of 1 to 7 weeks in the onset of high viremia compared to controls (Fig. 1C, dotted lines). Significant protection was afforded by PGT121 against the cell-associated SHIV challenge over the first 4 weeks

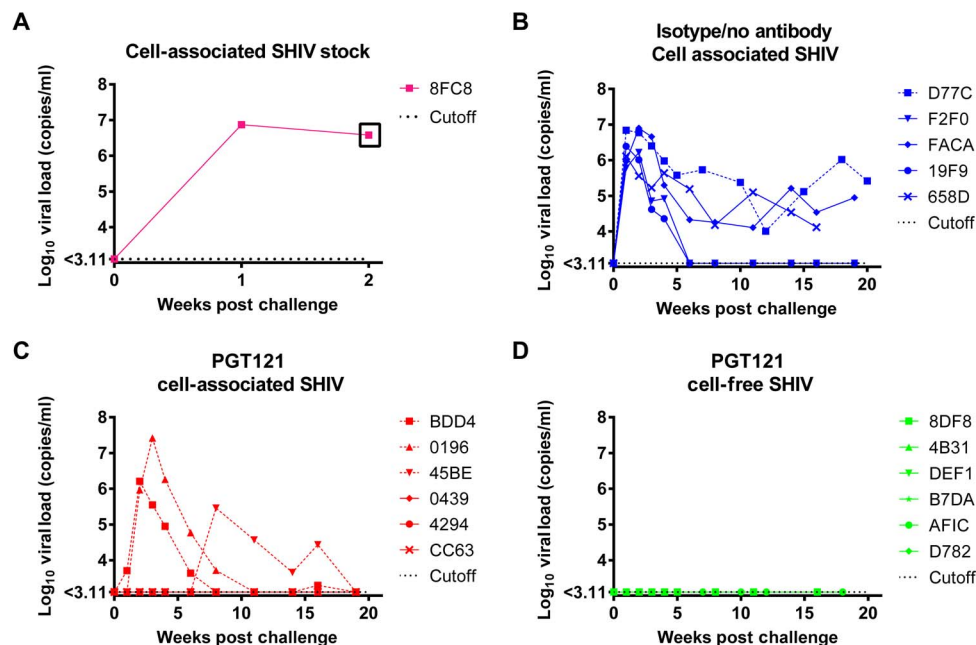


Fig. 1. Establishment of cell-associated SHIV_{SF162P3} stock and challenge of macaques infused with PGT121 or isotype control. (A) A SHIV_{SF162P3}-naïve pigtail macaque was challenged with cell-free virus via the intravenous route. The graph depicts the animal's plasma VL over 2 weeks after challenge. The time point enclosed by the block reflects the 2-week time point when the animal was euthanized, and its splenocytes were harvested to serve as a challenge stock of cell-associated virus against animals not infused with antibody or infused with an immunoglobulin 1 (IgG1) isotype control antibody ($n = 5$) or animals infused with the PGT121 BnAb ($n = 6$). (B) The graph depicts the VLs of animals not infused with antibody (dotted blue line) or infused with IgG1 isotype control (solid blue lines) 1 hour before challenge with cell-associated SHIV_{SF162P3}. (C) The VLs of PGT121-infused macaques that remained uninfected (solid red lines) or became infected (dotted red lines) after cell-associated virus challenge are depicted in the graph. (D) An additional six macaques were infused with PGT121 1 hour before being intravenously challenged with cell-free SHIV_{SF162P3}. The postchallenge VLs of these six animals are depicted in the graph (solid green lines). The dotted black lines in all graphs represent the sensitivity cutoff for the VL assay used.

(TWA VL difference = $1.79 \log_{10}$ copies/ml, $P = 0.015$; fig. S1B). These results demonstrate substantial but partial efficacy of PGT121 against this cell-associated virus challenge.

To evaluate whether pigtail macaques, like rhesus macaques (3), were protected from a cell-free SHIV_{SF162P3} challenge, we infused an additional six pigtail macaques with the same dose of PGT121 and challenged them intravenously with 874 TCID₅₀ (50% tissue culture infectious dose) of cell-free SHIV_{SF162P3}. The same amount of this virus stock was previously used to infect both the splenocyte donor macaque intravenously (shown in Fig. 1A) and eight naïve pigtail macaques mucosally (29). A comparison of the cell-free and cell-associated SHIV_{SF162P3} challenge inocula used is illustrated in table S1. All six PGT121-infused macaques were protected from viremia after the cell-free SHIV_{SF162P3} challenge (Fig. 1D), with significant protection observed compared to previous control animals challenged with the same virus mucosally (TWA VL difference = $2.71 \log_{10}$ copies/ml, $P = 0.0007$; fig. S1C).

SHIV-specific antibody responses after challenge

To analyze protection further, we first assessed whether anti-HIV-1 gp41 antibodies were generated after SHIV_{SF162P3} challenge. We chose to detect antibodies against the gp41 portion of HIV-1 envelope because measurements of this specificity would not be confounded by the presence of infused PGT121 within plasma samples from the experimental group. All five no antibody/isotype control antibody-infused animals developed anti-HIV-1 gp41 antibodies within 3 weeks (Fig. 2A). None of the PGT121-infused animals without detectable viremia developed gp41-specific antibodies (Fig. 2, B and C). Although the two animals exhibiting a 1-week delay before high viremia seroconverted to gp41 at similar time points after challenge as no antibody/isotype control-infused animals, the animal exhibiting delayed viremia until week 8 after challenge did not seroconvert to gp41 until week 8 (Fig. 2B). These results are consistent with the VL data and support the partial protection observed in the PGT121-infused animals. The three PGT121-infused animals with breakthrough SHIV infection ultimately controlled viremia; however, eventual control of SHIV_{SF162P3} viremia is common in macaques (30). gp120-binding antibody responses or the capacity of these antibodies to bind FcγRs were similar between animals controlling or not controlling viremia (figs. S5 and S6).

PGT121 antibody concentrations after infusion and relationship to viremia

The delay in the onset of viremia observed in three of the PGT121-infused animals was intriguing. To probe this further, we analyzed plasma anti-HIV-1 gp120 antibodies by ELISA to determine the concentration of PGT121 before the occurrence of viremia. Previous data in rhesus macaques showed a substantial decline in PGT121 at this dose between 1 and 4 weeks after infusion (3). In a subset of animals tested at 30 min after antibody infusion, high concentrations of PGT121 were detected (Fig. 3A). By 7 days after infusion, PGT121 was detected in all infused animals, but with a range of antibody concentrations (0.3 to 4.0 μg/ml; Fig. 3B). The two PGT121-infused animals that showed only a 1-week delay before high viremia had the lowest plasma concentrations of PGT121 (that is, 0.4 and 0.3 μg/ml). To assess what may constitute a protective amount of PGT121 against cell-associated SHIV, we directly measured concentrations of PGT121 30 min after infusion in a subset of animals. We estimated an average decay rate between 30 min and 1 week after infusion for these animals, which was used to extrapolate PGT121 concentrations from 1 week after infusion to 30 min for the other animals. PGT121 concentrations

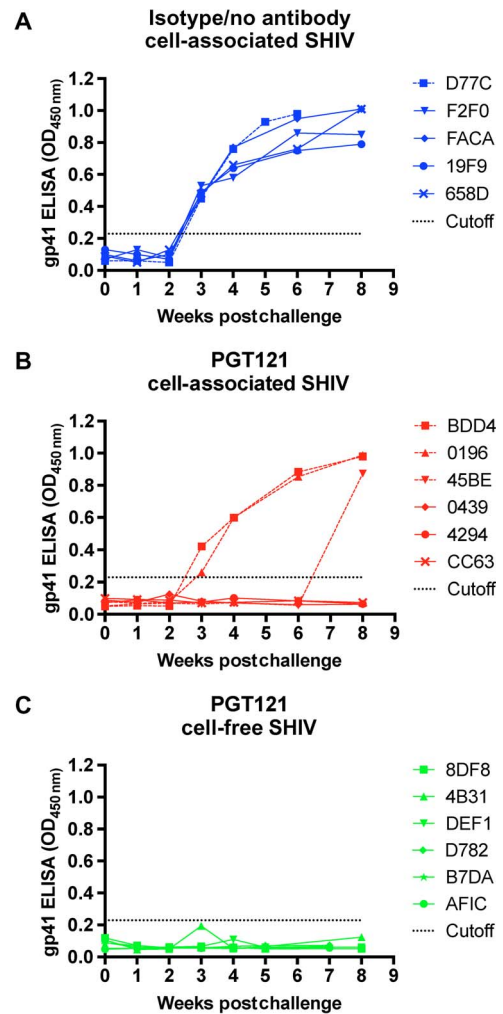


Fig. 2. Seroconversion of macaques challenged with cell-associated or cell-free SHIV_{SF162P3}. An enzyme-linked immunosorbent assay (ELISA) was used to screen serial plasma samples from no antibody/isotype control- or PGT121-infused animals challenged with cell-associated or cell-free SHIV_{SF162P3} for the presence of antibodies against the gp41 portion of viral envelope. The relative ODs for 1:1000 dilutions of plasma samples from the first 8 weeks after challenge are depicted in the graphs for (A) the no antibody/isotype control-infused macaques challenged with cell-associated virus, (B) PGT121-infused animals challenged with cell-associated virus, and (C) PGT121-infused animals challenged with cell-free virus. The dotted black line on all graphs represents the cutoff OD for the ELISA, which was defined as three times the OD of gp41-coated wells with a SHIV_{SF162P3}-uninfected plasma sample.

≥ 4.25 μg/ml at 30 min after infusion were fully protective against infection or, in the case of the animal with the 7-week delay before viremia (that is, animal 45BE), prolonged the duration before onset of viremia (Fig. 3A). Plasma PGT121 concentrations ≤ 1.77 μg/ml were not protective (Fig. 3A). Note that the plasma PGT121 concentration in animal 45BE had dropped from 2.5 μg/ml at week 1 to 0.7 μg/ml by week 6 after infusion.

Cell transfer studies of PGT121-mediated protection from SHIV

The delay in the onset of viremia observed in one of the PGT121-infused animals until antibody concentration had waned to less than 1 μg/ml was interesting. This suggested the possibility that virus-infected cells, either donor-derived or infected autologous cells resulting from

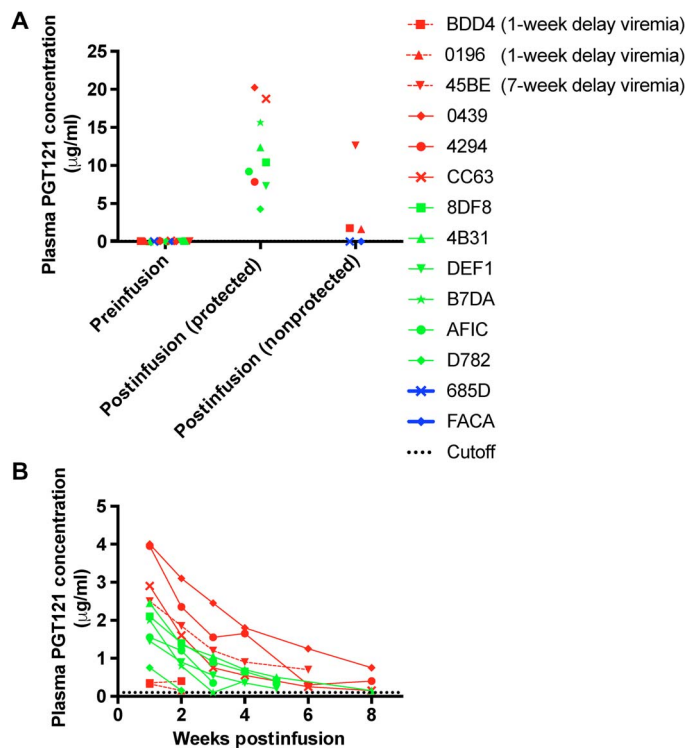


Fig. 3. Plasma concentrations of infused PGT121 BnAb. An ELISA to detect gp120-binding antibodies was used to screen preseroconversion serial plasma samples from animals infused with PGT121 (red or green data points) or isotype control antibodies (blue data points) and determine plasma concentrations of PGT121. (A) The graph depicts the relative amounts of PGT121 antibody in macaques infused with PGT121 or isotype control antibody at the week 0 time point (preinfusion) before antibody infusion and 30 min after antibody infusion (postinfusion) in protected and non-protected animals. Actual measurements of plasma PGT121 at 30 min after infusion were available for six protected animals (4294, CC63, 8DF8, B7DA, AFIC, and D782). For the other six animals (O196, BDD4, 45BE, O439, 4B31, and DEF1), the postinfusion PGT121 concentrations were extrapolated from measured week 1 postinfusion concentrations using the average rate of decay between 30 min and 1 week after infection in the six animals for which early postinfusion concentrations were measured. (B) The graph depicts the relative plasma concentrations of PGT121 at detectable preseroconversion serial time points for all 12 animals infused with PGT121.

cell-to-cell transfer, persisted during this delay and then initiated spreading infection after the PGT121 waned to a suboptimal concentration. We first speculated that inadvertent major histocompatibility complex (MHC) matching of the donor to some recipients may have allowed donor cells to persist for longer and initiate a delayed infection. Deep sequencing-based MHC genotyping of the animals, however, did not reveal any MHC matching of the donor to particular recipients (Table 1). We then surmised that very low amounts of plasma SHIV RNA or cellular SHIV RNA or DNA within PBMC may be detectable in stored blood samples, accounting for the recrudescence of infection. Using sensitive, well-validated quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) and PCR assays (31–33), we did not detect evidence of SHIV infection in blood samples between 1 and 4 weeks after exposure in the animal with prolonged delay in infection (table S2). We did observe, however, cell-associated viral DNA and RNA in PBMC, but not plasma RNA, at 6 weeks after challenge.

We then postulated that SHIV infection below the limits of detection, even in sensitive assays, might activate immune cells or stimulate SHIV-specific T cell responses, similar to observations in some cohorts of “HIV-

Table 1. MHC haplotypes of cell-associated SHIV donor and PGT121-infused macaques.

	Donor	Recrudescent virus		No recrudescent virus				
	Animal	8FC8	O196	45BE	BDD4	4294	O439	CC63
A1	A052	A082	A052	A052	A052	Unknown	A053	
A2	Unknown	A052	A082	A040	A019	A082	A082	
B1	B101	B015b	B004b	B004b	B004b	B007	B019	
B2	B007	B024	B118c	B118c	B013	B118b	B118b	
DRB1	DR105	DR05	DR12b	DR12b	DR12b	DR108	DR18	
DRB2	DR108	DR106	DR106	DR123	DR100	DR110	DR110	

exposed but uninfected” subjects (34, 35). We first analyzed stored PBMC samples from PGT121-protected animals for ex vivo expression of the HLA-DR activation marker on T cells and CD107a degranulation marker on natural killer (NK) cells but found no consistent pattern of enhanced expression of these markers in PGT121-protected animals (fig. S7). We also stimulated thawed PBMC with overlapping SIV Gag peptides and measured SIV-specific T cell responses by intracellular cytokine staining. Although low but detectable responses were present in infected animals, we could not detect Gag-specific T cell responses in protected animals (fig. S8).

To further analyze the possibility of a limited SHIV infection in apparently protected animals, we performed cell transfer studies using PBMCs stored from these animals before the onset of viremia. Hansen *et al.* (33) previously showed that PBMCs and/or lymph node mononuclear cells from unvaccinated rhesus macaques, which either control SIV viremia or have viremia controlled by antiretroviral therapy (ART), could readily transmit SIV infections to naïve rhesus macaques. We therefore transferred 2.0×10^7 to 2.3×10^7 PBMCs into six macaques that had previously been protected from cell-associated or cell-free viral challenge by PGT121 infusion (Fig. 1). These animals exhibited no previous evidence of SHIV_{SF162P3} infection and neither humoral nor cellular immune responses to SHIV_{SF162P3} (Fig. 2 and fig. S8). For a positive control, we first transferred PBMC from a controller macaque, which was SHIV_{SF162P3}-infected but controlled viremia after 4 weeks (Fig. 4A). Transfer of these PBMCs (obtained after viremia was controlled) readily initiated an infection within 1 week, similar to the results obtained by Hansen *et al.* (33) using elite controller SIV-infected rhesus macaques. We then transferred PBMCs from two animals completely protected by PGT121 from either cell-associated or cell-free SHIV_{SF162P3} challenge (Fig. 4, B and C). These transferred PBMCs did not initiate SHIV_{SF162P3} infections. We also transferred PBMCs (stored 1 week after SHIV exposure) from an animal with a 1-week delay before high viremia into an uninfected animal (Fig. 4D). An infection was initiated by these PBMCs in the uninfected animal with high viremia by 1 week after PBMC transfer. Finally, we studied PBMC transfer from the animal with a 7-week delay in viremia (animal 45BE). We transferred 2.2×10^7 PBMCs obtained from weeks 1 to 4 after SHIV challenge into two uninfected macaques (Fig. 4, E and F). No viremia was detected in the two recipients. Overall, the lack of SHIV sequences or ability to passage SHIV viruses from blood samples in 4 weeks after cell-associated SHIV_{SF162P3} challenge in animal 45BE suggests a very limited infection confined to tissues.

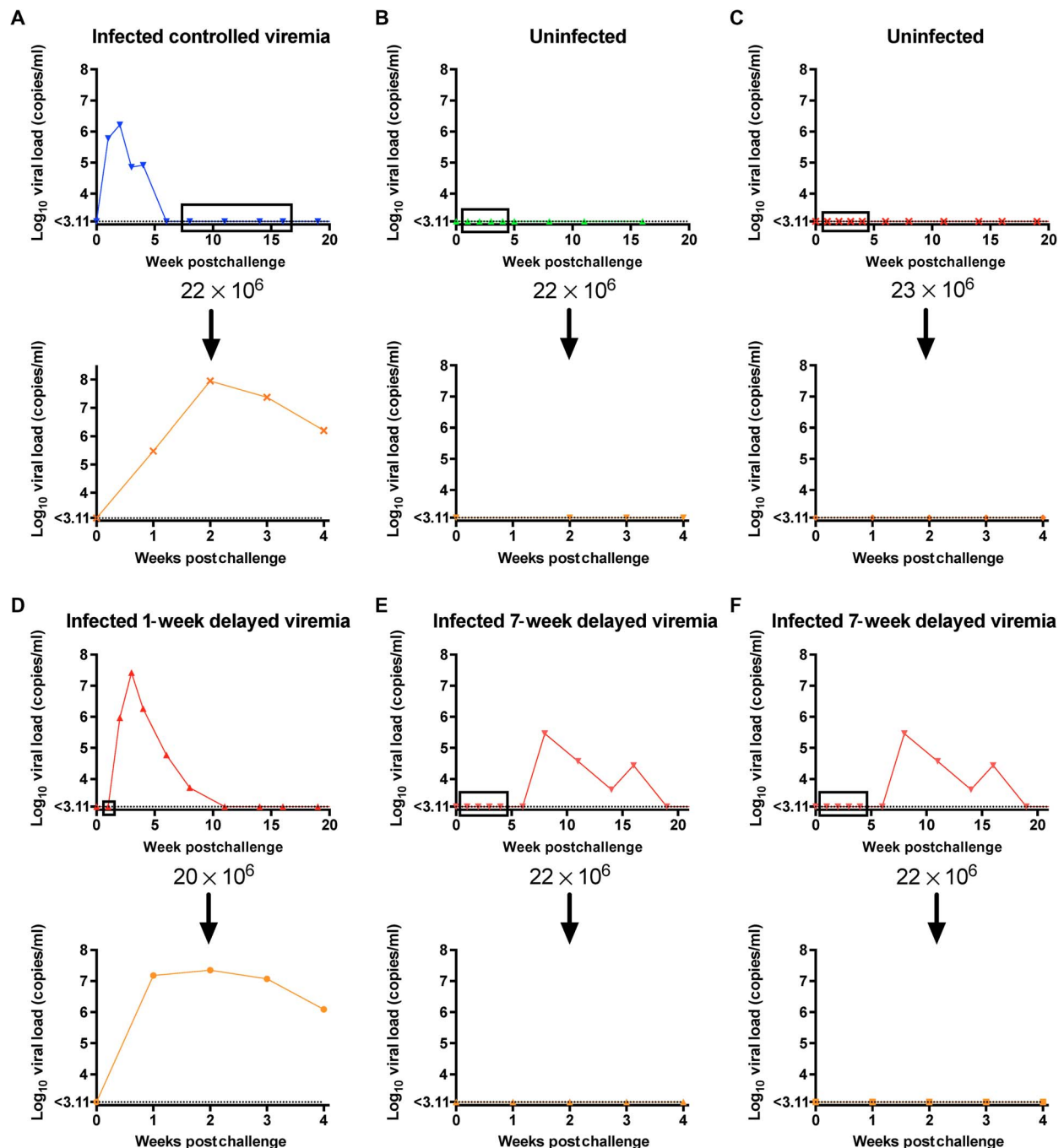


Fig. 4. Assessment of infectiousness of SHIV_{SF162P3}-challenged animals by transfer of PBMCs to uninfected macaques. To assess the infectiousness of several animals after challenge, about 20×10^6 PBMCs were injected intravenously into uninfected macaques. Newly challenged macaques were followed for viremia. (A to F) The pairs of graphs show the VLs of the PBMC source animals at the time points used for the challenge (time points in black boxes in top graphs) and VLs after PBMC challenge in the intravenously challenged animals (bottom graphs). (A) This graph represents transfer of PBMCs from an infected animal with controlled viremia (F2F0) into an uninfected animal (CC63). (B and C) These graphs depict transfers of PBMCs from two uninfected animals, 4B31 (B) and CC63 (C), into two additional uninfected animals (8DF8 and 4B31, respectively). (D) The graph shows the transfer of PBMCs from an infected animal (O196), from a time point before appearance of high viremia, to an uninfected animal (4294). (E and F) These graphs depict transfers of PBMCs from an animal with prolonged delay in viremia (45BE) into two uninfected animals, DEF1 (E) and O439 (F). (A to F) The dotted black lines in all graphs represent the sensitivity cutoff for the VL assay used.

Analysis of SHIV viruses in breakthrough animals

Three animals developed SHIV infection after the cell-associated SHIV exposure despite the PGT121 infusion. This raised the possibility that there may be a selection for particular variants of SHIV_{SF162P3} or

PGT121-resistant viruses. We therefore generated a mean of 27 (range, 20 to 29) single-genome SHIV_{SF162P3} Env clones from each of these three infected animals and a series of control animals, including the splenocyte donor animal, from plasma RNA obtained 2 weeks after

infection. The cell-associated SHIV_{SF162P3} stock derived from the donor animal was diverse as expected, given that it had undergone multiple *in vivo* passages (36). We identified 17 distinct variants in the 29 clones obtained. Next, we used the frequency distribution of these 17 variants to obtain an extrapolated number of 46 variants, had an even larger number of clones been generated [95% confidence interval (CI), 23 to 157; Fig. 5]. The diversity of SHIV_{SF162P3} early after infection remained high as expected, with a mean of 25.6 (range, 15 to 30.4) estimated distinct founders. However, viral diversity was lower within the two animals infused with PGT121 that exhibited viremia with a short delay after challenge, with a mean of 10.3 (range, 7.9 to 12.6) founders. Last, in the animal exhibiting a 7-week delay (animal 45BE) before exhibiting viremia, we observed only one viral variant in the 20 clones sequenced, consistent with a very limited initial infection and the long delay before the emergence of infection.

To determine whether there was a sieving for particular viral variants in the breakthrough animals, we analyzed Env sequences from the clones obtained. We found no pattern to suggest the emergence of a common SHIV_{SF162P3} variant in the three breakthrough animals. In

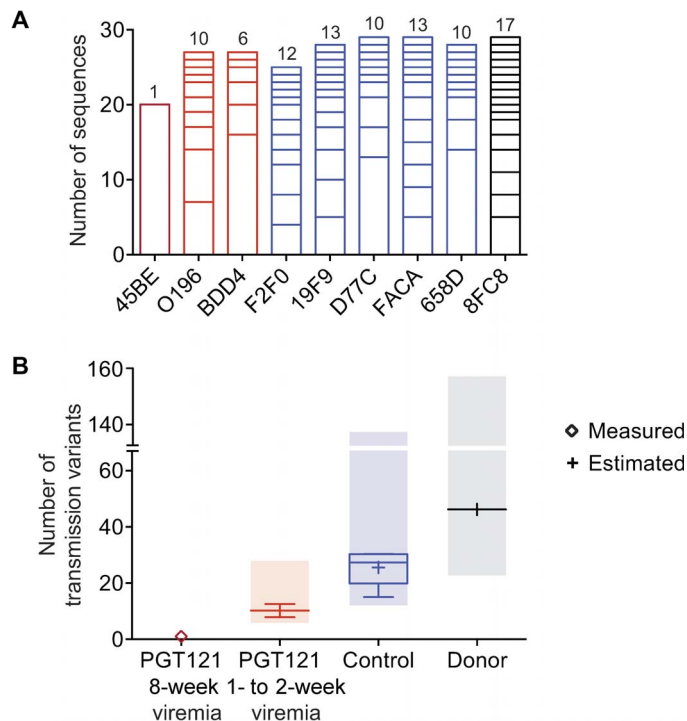


Fig. 5. Diversity of envelope transmission variants. (A) Distribution in the number of sequences observed for each transmission variant (represented by a segment of the bar chart), as determined by phylogenetic analysis of the Env region sequences. The number of different transmission variants observed for each animal is shown above each bar. (B) Distribution in the estimated total number of transmission variants across four groups of animals: PGT121-infused animal with no viremia until week 8 (45BE), PGT121-infused animals with 1- to 2-week viremia (O196 and BDD4), control animals (F2F0, 19F9, D77C, FACA, and 658D), and the donor animal (8FC8). Extrapolated diversities were calculated on the basis of the distribution of the observed multiple variants. The interquartile range, minimum and maximum (box-and-whisker plots), and mean (+) for the estimated diversity for each group of animals are shown. The shaded boxes represent the minimum lower limit and maximum upper limit of the 95% CI for the estimated diversity across all animals per group.

particular, across the V3 loop where PGT121 binds, there were minimal or no sequence changes observed (fig. S9). Outside the V3 loop in Env, there was only one amino acid change (at position 823, distant to the V3 loop) common to the five control animals and absent in all three breakthrough animals. However, the frequency of this change was not significantly different between control and breakthrough animals when corrected for multiple comparisons (table S3).

The growth of SHIV_{SF162P3} in the presence of low concentrations of PGT121 might result in the selection of PGT121-resistant viruses, although we did not observe sequence changes to suggest that this was likely. To formally assess for the selection or generation of PGT121-resistant SHIV_{SF162P3} isolates in the three animals with breakthrough SHIV_{SF162P3} infection, we generated a pseudotyped Env virus from common variants within the donor animal and each of the three breakthrough animals (Table 2). We found that the cell-associated SHIV_{SF162P3} challenge stock was highly sensitive to PGT121 neutralization as expected. The clones derived from the three breakthrough animals remained neutralization-sensitive to PGT121, suggesting that PGT121 resistance was not the cause of the loss of viral control in these animals. As controls for this neutralization assay, we also used the V1V2-specific PG9 BnAb, which does not neutralize SHIV_{SF162P3} (37–39), and a combination of the variable loop-specific CH01 and CD4 binding site-specific CH31 antibodies (40, 41) as positive internal controls.

DISCUSSION

There is considerable focus on generating or delivering anti-HIV-1 BnAbs to prevent HIV-1 infections. We confirmed that the promising BnAb PGT121 is fully protective against a high-dose intravenous cell-free SHIV challenge of pigtail macaques, similar to observations with intravaginal cell-free virus challenge in rhesus macaques (3). There are conflicting reports, depending on the *in vitro* model used, on the capacity of PGT121 to prevent cell-to-cell HIV-1 transmission *in vitro* (23, 24). We found that PGT121 was only partially efficacious at preventing infection after a high-dose intravenous cell-associated SHIV_{SF162P3} challenge. Although we acknowledge that the cell-associated SHIV challenge used was designed to test the limits of protection (that is, 24.5 million cells from an animal with acute SHIV infection infused intravenously), this raises concerns that BnAbs, even when the virus is sensitive to the antibody, may not prevent all cell-associated HIV-1 transmissions.

The pattern of protection observed was instructive. In two animals preinfused with PGT121 and exposed to cell-associated SHIV, a delay of 1 week in the occurrence of high viremia was observed, as compared to isotype or non-antibody-treated controls. These two animals had the two lowest concentrations of PGT121 across the infused animals, suggesting that the amount of BnAb available *in vivo* is likely to be important in protection from cell-associated SHIV. Our data on the protective concentrations of PGT121 against cell-associated SHIV are broadly consistent with the titration studies of Moldt *et al.* (3) for protection against cell-free SHIV in rhesus macaques, where concentrations of PGT121 (≤ 1.8 $\mu\text{g/ml}$) were only partially protective. The similarities in the protection profiles are consistent with the early control of most infected cells in most macaques challenged with cell-associated SHIV and the neutralization of any free SHIV produced from the infused cells (or infected host cells) *in vivo*.

The delay in one PGT121-infused animal for 7 weeks before the detection of viremia was concerning. The animal had similar initial concentrations of circulating PGT121 as other protected animals, but PGT121 in the circulation had waned to 0.7 $\mu\text{g/ml}$ by 6 weeks, just

Table 2. Neutralization sensitivity of breakthrough viruses. IC₅₀, 50% inhibitory concentration.

	IC ₅₀ (µg/ml) in TZM-bl cells				
	SHIV _{SF162P3} cell-associated virus stock (donor animal 8FC8)	SHIV _{SF162P3} breakthrough virus at 8 weeks (animal 45BE)	SHIV _{SF162P3} breakthrough virus at 1 week (animal 0196)	SHIV _{SF162P3} breakthrough virus at 1 week (animal BDD4)	SHIV _{SF162P3} virus stock
PGT121	0.03	0.03	0.02	0.02	0.09
PG9	>10	>10	>10	>10	>10
CH01-31	0.47	0.45	0.37	1.03	2.74

before the detection of the delayed viremia. We initially hypothesized that low numbers of circulating infected cells, derived from either the donor splenocytes in the challenge stock or host cells infected in a cell-to-cell manner from the donor splenocytes, may have persisted. Once the PGT121 had declined to a suboptimal concentration, these infected cells may have initiated a spreading infection. Detailed study of stored blood samples did not detect evidence of SHIV virus or immune activation during weeks 1 to 4 after challenge. However, SHIV infection did become detectable at 6 weeks after challenge via cellular viral RNA and DNA but not plasma viral RNA. Further, cell transfer of PBMCs collected before viremia from this animal to two uninfected recipients failed to initiate an infection. This method to initiate an infection is likely to be highly sensitive because we showed that where viremia was not detectable with standard assays (one controller and one just before the onset of viremia), this method initiated a SHIV infection within 1 week. Further, PBMCs from both elite controllers and ART-treated SIV-infected rhesus macaques without viremia readily initiate SIV infections when transferred to uninfected animals (33). We speculate that SHIV-infected cells may have been seeded into tissues of this animal after the cell-associated SHIV challenge and laid dormant until the PGT121 concentrations waned weeks later. This hypothesis is consistent with the single founder identified in this animal and the retention of PGT121 neutralization sensitivity of the recrudescence strain. Whether this is a rare phenomenon and peculiarly related to the high-dose cell-associated challenge we used requires further study. Nonetheless, in principle, this work suggests that HIV-1-specific BnAbs, either passively infused or induced by vaccination, may not always protect against cell-associated virus exposures *in vivo*.

We acknowledge several limitations of our work. The dose of BnAbs to protect against cell-associated compared to cell-free exposure may be different, and more precise dose titrations may help the goals of future clinical trials of BnAb-based prevention strategies. Our experiments used intravenous SHIV challenges that do not mimic most human HIV-1 exposures and may require different concentrations of BnAbs for protection. Our cell-associated challenge used splenocytes from a SHIV-infected macaque that does not mimic HIV-1-infected cells in genital secretions. Although we did not detect PGT121 resistance in viral variants tested from animals that had breakthrough infections despite the PGT121 infusion, we cannot exclude that some variants may have had reduced PGT121 sensitivity.

There is considerable evidence that Fc-dependent antibody functions are important in protection from HIV-1 and other viral infections such as influenza (2, 42–44). Fc-dependent antibody functions including ADCC may be especially important in controlling cell-associated virus exposure,

because unlike cytotoxic T lymphocytes, ADCC can recognize foreign MHC-mismatched infected cells and recruit innate immune cells to kill infected cells and liberate cytokines (45). Direct analyses of the importance of Fc-dependent functions of antibodies against cell-associated SHIV infections are now feasible with this model using Fc-mutant PGT121 or other BnAbs. Although we found that the PGT121 BnAb can mediate ADCC *in vitro* using macaque effector cells, there was considerable variability in the capacity of effector cells from our outbred macaques. This is similar to the ADCC potential of NK cells observed in humans, where roles for the education of NK cells by inhibitory receptor/ligand combinations and NK cell differentiation influence antibody-dependent functions (46–49). We speculate that, for improved protection against cell-associated SHIV infection, it may be critical to deliver combinations of BnAbs with enhanced Fc-dependent functions (50) and/or improve the ability of effector cells to mediate efficient ADCC.

MATERIALS AND METHODS

Study design

Our goal was to analyze the protective capacity of the BnAb PGT121, compared to an isotype control antibody, against a cell-associated infection using a preclinical macaque SHIV_{SF162P3} challenge model. On the basis of previous studies of PGT121 and other BnAbs against cell-free virus challenges, we predicted that the PGT121 would either completely prevent infection or result in rapid high viremia in pigtailed macaques. Our previous work in pigtailed macaques infected mucosally with cell-free SHIV_{SF162P3} (29) provided an estimate that we would have 80% power ($\alpha = 0.05$) to detect a difference (1.07 log₁₀ copies/ml) in mean TWA VL over the first 4 weeks of infection (the typical duration of high viremia in control animals) using six animals per group. Primary data are located in table S4.

Animals

Juvenile male pigtailed macaques were purchased from the Australian National Breeding Colony. cDNA from PBMC was genotyped for MHC alleles as previously described (51). The Australian Commonwealth Scientific and Industrial Research Organization Animal Health Animal Ethics Committee approved all macaque studies.

PGT121 and isotype control antibody infusions

Theraclone Sciences Inc. provided the PGT121 stock at 8.7 mg/ml in phosphate-buffered saline (PBS) + 0.02% Tween. MP Biomedicals provided human IgG1 isotype control purified from human myeloma plasma, which was negative for antibodies to HIV-1 and HIV-2. The

isotype control antibody was provided at 2.95 mg/ml in PBS + 0.02% Tween. Four/five macaques were infused with IgG1 isotype control (1 mg/kg, iv) before being challenged with cell-associated SHIV_{SF162P3}. Twelve macaques were infused with PGT121 (1 mg/kg) before being challenged with either cell-associated SHIV_{SF162P3} or cell-free SHIV_{SF162P3} (six animals each).

Cell-associated challenge stock and cell-associated and cell-free SHIV_{SF162P3} challenges

We first infected one pigtailed macaque intravenously with cell-free SHIV_{SF162P3} [supplied by the National Institutes of Health (NIH) AIDS Reagent Repository]. We used an amount of a stock previously found to successfully infect 24 pigtailed macaques intravaginally in an earlier vaccine study (29). A reanalysis of this stored cell-free stock showed that it retained a high TCID₅₀ and had a high amount of SHIV RNA (table S1). Two weeks later, at a time of high plasma SHIV_{SF162P3} RNA, we euthanized the animal, harvested the spleen, and made splenic mononuclear cell preparations, freezing the cells immediately at 4.9×10^7 cells per vial. An analysis of this cell-associated stock showed it to contain 4×10^5 cellular RNA copies and 3×10^3 DNA copies of SHIV_{SF162P3} per 10^6 splenocytes (table S1). To challenge a further 11 animals—6 of which had been infused with PGT121, and 5 of which had been infused with IgG1 isotype control antibody or no antibody—we thawed and infused 2.45×10^7 cells intravenously per animal. A limited in vivo intravenous titration of this infected splenocyte stock showed that 1:100 and 1:1000 dilutions were still infectious, but 1:10,000 and 1:100,000 dilutions were noninfectious, suggesting that the animal infectious dose administered was ~1000 (fig. S2).

The copy number of SHIV_{SF162P3} RNA per milliliter of plasma was measured on serial blood samples using quantitative real-time RT-PCR, as previously described (52). Briefly, RNA was purified from plasma and reverse-transcribed. Quantification of viral RNA copies within plasma was accomplished using a TaqMan minor groove binding 5' fluorescently labeled probe and a 3' nonfluorescently labeled quencher. To probe the limits of the protection observed, a subset of plasma samples was also assessed for SHIV RNA using a quantitative RT-PCR assay for an SIV gag target sequence with a detection limit of 15 copies/ml, as detailed elsewhere (32), and a subset of PBMC samples was assessed for cellular SHIV DNA and SHIV RNA using nested quantitative PCR and RT-PCR methods for quantification of an SIV gag target sequence, as described previously (31, 33). To confirm the ability of PGT121 to protect against cell-free SHIV_{SF162P3}, an additional six animals were infused with PGT121 and challenged intravenously with the same dose of cell-free virus that was used to infect the source of the cell-associated stock.

ELISA-based analyses of seroconversion to SHIV_{SF162P3} and plasma concentrations of PGT121

ELISAs were performed using a modification of a previously reported protocol (53). To assess seroconversion to the SHIV challenge, we coated plates with HIV-1 gp41 (200 ng per well) (ProSpec) in carbonate-bicarbonate coating buffer (Sigma-Aldrich) overnight at 4°C. HIV-1 gp41 was implemented as the capture antigen because it should detect antibodies arising from SHIV_{SF162P3} infection but not infused PGT121, which binds to gp120. The following day, plates were washed thrice with wash buffer (PBS + 0.1% Tween 20) and blocked with blocking buffer [PBS + 0.1% Tween 20 + 5% bovine serum albumin (BSA)] for 1 hour at 37°C. Plates were then washed thrice, and serial macaque plasma samples from week 0 (before challenge) to week 8 after challenge were

added in a log₁₀ dilution series (1:100 to 1:100,000) to plates in duplicate in dilution buffer (PBS + 0.5% NP-40 + 0.2% Tween 20 + 0.1% BSA) and incubated for 90 min at 37°C. After incubation, plates were washed six times with wash buffer and incubated for 1 hour with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (Invitrogen). Plates were washed six times and developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich). The development reaction was stopped with 1 M H₂SO₄, and plates were read with a plate reader at 450 nm. Samples were considered positive when the optical densities (ODs) of postchallenge samples reached at least three times the average OD of gp41-coated wells with SHIV-uninfected plasma samples added.

To determine the amount of PGT121 in plasma samples from animals infused with the monoclonal antibody, we performed an additional set of ELISAs with plates coated with HIV-1_{AD8} gp120 (200 ng per well). The HIV-1_{AD8} gp120 was prepared as previously described (54). These ELISAs differed from the seroconversion ELISAs in two additional aspects: (i) Plasma samples were added within dilution buffer at either a 1:25 or 1:50 dilution; (ii) a standard curve was created using a series of dilutions of PGT121, ranging from 10 to 0 µg/ml, in dilution buffer. For a subset of eight animals (that is, six that received PGT121 and two that received isotype control), we studied plasma samples 30 min after infusion. All additional plasma samples were collected at the same time points used to assess VLs. All additional aspects of these ELISAs were conducted as per the seroconversion ELISA protocol above. For animals lacking a 30-min postinfusion time point sample, we estimated the plasma PGT121 concentrations. We assumed exponential decay in concentration from time of infusion to week 1 after infusion. The PGT121 concentration measurements at 30 min and 1 week after infusion for animals 4294, CC63, 8DF8, B7DA, AFIC, and D782 were used to determine an average decay rate (k_{av}). For each animal, initial concentration C_0 was estimated using k_{av} and the measured concentration at week 1 after infusion. The concentration at early (that is, $t = 30$ min) postinfusion time points was then estimated using $C(t) = C_0 \exp(k_{av}t)$. Variation between the six animals with measured 30-min postinfusion concentrations resulted in differences for some animals between the measured concentrations and those estimated using an average growth rate (that is, average absolute difference, 3.96 µg/ml; range in absolute differences, 0.22 to 12.13 µg/ml).

Cell transfer studies

To assess whether PBMCs from infected animals could transmit infection, we intravenously transferred about 2.0×10^7 to 2.2×10^7 PBMCs from time points before viral detection to uninfected animals. As negative controls, 2.2×10^7 to 2.3×10^7 PBMCs from two uninfected macaques, infused with PGT121 and challenged with either cell-associated SHIV_{SF162P3} or cell-free SHIV_{SF162P3}, were transferred to two additional uninfected macaques. As a positive control, 2.2×10^7 PBMCs from a SHIV_{SF162P3}-infected macaque with controlled viremia were transferred to an uninfected macaque. Serial blood samples from all challenged animals were used to measure SHIV_{SF162P3} plasma RNA copies, as described above.

Detection of immune complex binding to FcγRs by flow cytometry

The ability of PGT121 immune complexes to bind to human and macaque FcγRs was assessed using a previously described method (55). Briefly, PGT121 (0.6 µg/ml) was incubated with anti-human Fab'₂ conjugated with Alexa Fluor 647 (0.3 µg/ml) (Jackson ImmunoResearch Laboratories) to form immune complexes. Next, these complexes were incubated with the FcγR-deficient IIA.1.6 cell line transfected to express

human FcγRIIIa-Val¹⁵⁸/FcγR subunit or FcγRIIa (His¹³¹ variant) or the *Macaca nemestrina* FcγRIIIa/FcγR subunit or FcγRIIa (His¹³¹ variant). Untransfected cells were used as the negative control. Binding of immune complexes was assessed by flow cytometry.

Antibody-dependent NK cell activation assay

A modified version of an antibody-mediated NK cell activation assay previously used to detect macaque anti-influenza antibody-dependent NK cell activation was used to assess PGT121-triggered activation of macaque NK cells (56). Briefly, an ELISA plate was coated with HIV-1_{SF162} gp140 (600 ng per well) (obtained from the NIH AIDS Reagent Repository) overnight at 4°C. The ELISA plate was then washed with PBS and blocked with PBS + 5% BSA for 1 hour at 37°C, and then, wells were incubated for 2 hours at 37°C in the absence of antibody or presence of PGT121 (20 μg/ml). Next, the plate was washed with PBS, and 10⁶ freshly isolated macaque PBMCs were added to each well. Allophycocyanin-H7-conjugated anti-human CD107a antibody (clone H4A3; BD Biosciences), brefeldin A (5 μg/ml) (Sigma-Aldrich), and monensin (5 μg/ml) (BD Biosciences) were also added to each well. The plate was then incubated for 5 hours at 37°C with 5% CO₂. After incubation, cells were stained with Pacific Blue-conjugated anti-human CD3 (clone SP34-2; BD Biosciences), peridinin chlorophyll protein complex-conjugated anti-human CD8 (clone SK1; BD Biosciences), and allophycocyanin (APC)-conjugated anti-human NKG2A (clone Z199; Beckman Coulter) antibodies for 30 min at room temperature in the dark. Next, cells were fixed and acquired on an LSRFortessa flow cytometer (BD Biosciences). Samples were analyzed with FlowJo software.

Infected cell elimination/flow cytometry-based ADCC assay

A modified version of the infected cell elimination assay was performed to assess PGT121-mediated ADCC using macaque effector cells (57, 58). The HIV-1_{LAV}-infected 8E5/LAV cell line (obtained from the NIH AIDS Reagent Repository) was used as targets, and freshly isolated macaque PBMCs were used as effectors. Target 8E5/LAV cells were first stained with the LIVE/DEAD near-IR viability dye (Life Technologies) and the cell proliferation dye eFluor 670 (eBioscience), whereas effector cells were stained with the cell proliferation dye eFluor 450. Effector cells (10⁶) were mixed with target cells (2 × 10⁵) at a 5:1 ratio in the presence of PGT121 (20 μg/ml), human intravenous IgG (20 μg/ml), or no antibody. The cell mixture was centrifuged at 300g for 1 min before a 5-hour incubation with 5% CO₂ at 37°C. Cells were then fixed with 1% formaldehyde and permeabilized with 1× fluorescence-activated cell sorter permeabilization buffer (BD Biosciences). A phycoerythrin (PE)-conjugated antibody against HIV-1 p24 (KC57-RD1, Beckman Coulter) was then added to stain for HIV-1-infected target cells. Last, cells were fixed with 1% formaldehyde and acquired on an LSRFortessa flow cytometer (BD Biosciences). Percentage of ADCC was calculated using the following formula: %p24⁺ cells in [(targets + effectors) – (targets + effectors + Ab)]/(targets only).

ELISA to assess FcγRIIIa interaction with gp120-binding antibody

The interaction of anti-gp120 antibody in plasma from SHIV_{SF162P3}-infected animals with human FcγRIIIa was assessed using a modification of a previously described method (59). Briefly, ELISA plates were coated with HIV-1_{AD8} gp120 (50 ng per well) overnight at 4°C. The following day, plates were blocked with PBS containing 5% BSA at 37°C for 1 hour. Next, plasma samples from infected macaques were

added to the plates at log₁₀ dilutions. Bound IgGs were reacted with biotinylated dimeric recombinant soluble FcγRIIIa ectodomains followed by detection with HRP-conjugated streptavidin. ELISAs were developed with TMB substrate and stopped with 1 M H₂SO₄. Plates were read with a plate reader at 450 nm. Cutoff was defined as three times the OD obtained using a SHIV-uninfected plasma sample.

Ex vivo assessment of NK cell and T cell activation

To assess the activation status of NK cells and T cells after SHIV challenge directly ex vivo, cryopreserved PBMCs were surface-stained with fluorochrome-conjugated antibodies to CD3 (clone SP34-2; BD Biosciences), CD8 (clone SK1; BD Biosciences), NKG2A (clone Z199; Beckman Coulter), CD107a (clone H4A3; BD Biosciences), and HLA-DR (clone L243; BD Biosciences). Cells were then fixed with 1% formaldehyde and acquired on an LSRFortessa flow cytometer (BD Biosciences). Data were analyzed using FlowJo software. NK cells were defined as CD3⁻CD8⁺NKG2A⁺-gated lymphocytes and were assessed for CD107a expression. T cells were defined as CD3⁺-gated lymphocytes and were assessed for HLA-DR expression.

Detection of Gag-specific T cell responses

Gag-specific T cell responses were detected as previously described (60). Briefly, about 10⁶ cryopreserved PBMCs were incubated with overlapping SIV_{mac239} Gag 15-mer peptide pools (1 μg/ml) (obtained from the NIH AIDS Reagent Repository), the dimethyl sulfoxide (DMSO) peptide vehicle alone, or staphylococcal enterotoxin B for 5 hours at 37°C with 5% CO₂. After stimulation, cells were surface-stained with fluorochrome-conjugated antibodies to CD3 (Pacific Blue; clone SP34-2; BD Biosciences) and CD4 (fluorescein isothiocyanate; clone L200; BD Biosciences). Next, cells were fixed, permeabilized, and stained with fluorochrome-conjugated antibodies to IFNγ (APC; clone B27; BD Biosciences) and tumor necrosis factor (TNF) (Pe-Cy7; clone mAb11; BD Biosciences). Last, cells were fixed with 1% formaldehyde and acquired on an LSRFortessa. Data were analyzed with FlowJo software. Cutoff for positive responses of gated T lymphocytes expressing both IFNγ and TNF was set as three times the average response upon incubation in the presence of DMSO peptide vehicle alone.

Single-genome analyses and neutralization studies of infecting SHIV_{SF162P3} viruses

We analyzed the sequence diversity of infecting SHIV_{SF162P3} strains through single-genome sequencing using limiting dilution PCR as previously described (61, 62). We studied plasma RNA derived 2 weeks after challenge or at the appearance of viremia, if later, given there is likely to be minimal evolution at this early time point. Sequences for each animal were aligned, and the number of unique clones was identified. For the samples in which multiple variants were observed, the total diversity was calculated by extrapolation based on the distribution of the observed multiple variants. The extrapolated diversities were calculated using the Chao1 richness estimator for individual-based abundance data in EstimateS (version 9, R. K. Colwell, <http://purl.oclc.org/estimates>). This method reports a mean and 95% CI for the estimated number of variants (that is, mean and 95% CI among resampling runs). For one of the samples (658D), which contained no doublet sequences in the sample, the bias-corrected formula for the Chao1 richness estimator was used. Common variants of Env clones in the donor animal and the three PGT121-infused animals exhibiting breakthrough infections were cloned in pseudotyped expression vectors and assessed

for neutralization sensitivity against PGT121 and control monoclonal antibodies, as previously described (63).

Statistics

Statistical differences between TWA log₁₀ plasma VLs were assessed by two-sided Mann-Whitney tests. For these tests, a 0.05 α level was implemented. The statistical significance of differences in mutations in Env between PGT121-treated animals with breakthrough infections and SHIV-infected control animals was assessed by using two-sided Fisher's exact tests with a 0.05 α level. Multiple comparisons were corrected using the Bonferroni correction.

SUPPLEMENTARY MATERIALS

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- Fig. S1. Analysis of differences in VLs after different SHIV challenges.
 Fig. S2. Animal infectiousness of the cell-associated SHIV_{SF162P3} stock challenge dose.
 Fig. S3. PGT121 binds to macaque Fc γ Rs and activates NK cells.
 Fig. S4. ADCC of HIV-1-infected target cells by macaque PBMCs using PGT121.
 Fig. S5. Anti-gp120-binding antibodies in plasma samples of SHIV_{SF162P3}-infected macaques.
 Fig. S6. Fc γ R1-binding antibodies in plasma samples of SHIV_{SF162P3}-infected macaques.
 Fig. S7. Markers of NK cell and T cell activation in PGT121-infused animals.
 Fig. S8. Gag-specific T cells in SHIV_{SF162P3}-challenged macaques.
 Fig. S9. V3 loop sequence alterations in infected animals.
 Table S1. Characteristics of viral challenge stocks.
 Table S2. Sensitive VL measurements in PGT121-infused cell-associated challenged macaques.
 Table S3. Mutations within envelope.
 Table S4. Primary data.

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Partial efficacy of a broadly neutralizing antibody against cell-associated SHIV infection

Matthew S. Parsons, Sarah B. Lloyd, Wen Shi Lee, Anne B. Kristensen, Thakshila Amarasena, Rob J. Center, Brandon F. Keele, Jeffrey D. Lifson, Celia C. LaBranche, David Montefiori, Bruce D. Wines, P. Mark Hogarth, Kristine M. Swiderek, Vanessa Venturi, Miles P. Davenport and Stephen J. Kent

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Antibodies circumvented by cell-associated virus

Antibodies that are able to neutralize a range of HIV-1 strains are being developed to prevent infection in humans. Preclinical testing of these antibodies predominantly uses free virus during the challenge, although natural HIV-1 transmission could involve transfer of infected cells. To determine whether antibodies would still be able to combat this type of transmission, Parsons *et al.* developed a challenge model in nonhuman primates using SHIV-infected splenocytes. A well-characterized anti-HIV-1 antibody failed to protect all of the animals from the cell-associated viral challenge, although it had protected all animals from cell-free viral challenge. Their findings indicate that sustained high concentrations of circulating antibody may be necessary for prevention of cell-associated HIV-1 acquisition.

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