# Fc-dependent functions are redundant to efficacy of anti-HIV antibody PGT121 in macaques

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**A considerable body of evidence suggests that Fc-dependent functions improve the capacity of broadly neutralizing antibodies (BnAbs) to protect against and control HIV-1 infection. This phenomenon, however, has not been formally tested in robust cell-associated macaque simian-human immunodeficiency virus (SHIV) models with newer-generation BnAbs. We studied both the WT BnAb PGT121 and a LALA mutant of PGT121 (which has impaired Fc-dependent functions) for their ability to protect pigtail macaques from an i.v. high-dose cell-associated SHIV$_{SF162P3}$ challenge. We found that both WT and LALA PGT121 completely protected all 12 macaques studied. Further, partial depletion of NK cells, key mediators of Fc-dependent functions, did not abrogate the protective efficacy of PGT121 in 6 macaques. Additionally, in animals with established SHIV$_{SF162P3}$ infection, SHIV viremia levels were equally rapidly reduced by LALA and WT PGT121. Our studies suggest that the potent neutralizing capacity of PGT121 renders the Fc-dependent functions of the Ab at least partially redundant. These findings have implications for Ab-mediated protection from and control of HIV-1 infection.**

## Introduction

Passive administration of broadly neutralizing antibodies (BnAbs) reliably protects nonhuman primates from chimeric simian-human immunodeficiency virus (SHIV) challenge. A range of BnAbs delivered via either i.v. or mucosal routes consistently protect macaques from high-dose and serial low-dose mucosal challenges with multiple SHIV strains (1–4). The success of passive immunization in animal models has resulted in the initiation of human clinical trials assessing the capacity of the VRC01 BnAb to prevent HIV-1 infection (5).

A potential caveat to the notion that BnAbs are an ideal means of preventing new HIV-1 infections is the ability of these Abs to neutralize cell-associated virus. Cell-associated virus is highly infectious in the nonhuman primate SIV model of HIV-1 infection (6, 7), and both cell-free and cell-associated viruses appear to be capable of initiating human HIV-1 infections (8). The infectious nature of cell-associated virus is concerning, given that many BnAbs exhibit diminished neutralization capacity against cell-associated virus in in vitro assays (9, 10). We recently established a cell-associated SHIV infection model in pigtail macaques (11). Passive administration of the human PGT121 BnAb to macaques provided only partial protection from a very high-dose challenge

with cell-associated SHIV. Breakthrough infections appeared in 2 animals with insufficient PGT121 concentrations and in 1 animal with an apparent occult infection that revealed itself after waning of the passively administered Ab.

The mechanism or mechanisms utilized by PGT121 to protect against cell-associated virus challenge remain unknown. In rhesus macaque studies of free virus challenges, the b12 BnAb has been shown to function primarily through neutralizing free virions but to be assisted by its capacity to mediate Fc-dependent functions. Indeed, passive administration of a version of b12 mutated to diminish Fc-dependent functions (i.e., b12 LALA) partially abrogated protection from SHIV challenge (2). This partial abrogation was due to diminished Fc-dependent functions and not to the reduced complement fixation of the Ab, as a version of b12 mutated to diminish binding to complement (i.e., b12 KA), but not to Fc receptors (FcRs), conferred protection from SHIV challenge similar to that conferred by WT b12. Like b12, PGT121 binds FcRs and triggers Fc-dependent functions, such as Ab-dependent cellular cytotoxicity (ADCC) (12). We previously demonstrated that pigtail macaque NK cells degranulate upon exposure to PGT121-antigen complexes (11). Further, we observed that pigtail macaque effector cells could lyse HIV-1–infected target cells in a PGT121-dependent manner. In an in vivo environment, the Fc-dependent functions of BnAbs probably serve to eliminate newly infected cells established after exposure to cell-free virus. Indeed, viral DNA is detected at distal sites in otherwise "protected" animals for up to 1 week after cell-free SHIV challenge in PGT121-infused macaques (13). The number of infected cells at distal sites may be increased in PGT121-infused animals exposed to cell-associated virus, as the BnAb incompletely neutralizes cell-cell viral transmission (14). As
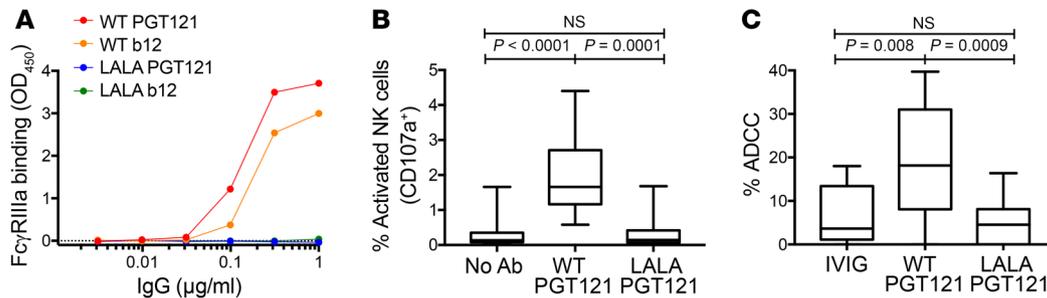
**Figure 1. Capacity of WT and LALA PGT121 to engage FcγRIIIa and mediate Fc-dependent functions in vitro.** (**A**) Graph depicts the relative ability of WT and LALA versions of PGT121 and b12 to engage pigtail macaque FcγRIIIa, as determined by ELISA. (**B**) Ab-dependent activation of macaque ($n$ = 16) NK cells through recognition of antigen-bound WT or LALA PGT121. NK cell activation was measured by flow cytometry as the percentage of NK cells expressing the degranulation marker CD107a after a 5-hour incubation in the presence or absence of WT or LALA PGT121 (20 μg/ml) bound to HIV-1$_{SF162}$ gp140 protein. (**C**) The capacity of WT and LALA PGT121 to facilitate ADCC was assessed using a flow cytometry–based infected cell elimination assay. ADCC against HIV-1$_{LAV}$–infected 8E5/LAV cells by macaque PBMCs ($n$ = 16) was measured in the presence of human IVIG (lacking anti–HIV-1 Abs), WT, or LALA PGT121 (20 μg/ml). Data were compared using a Friedman test followed by Dunn's post hoc tests. $P < 0.05$ was considered statistically significant. Box-and-whisker plots display the median (horizontal line within the box), IQR (top and bottom edges of the box), and range (horizontal lines on whiskers) of each condition.

such, we hypothesized that Fc-dependent functions of PGT121 play an important role in protecting macaques from cell-associated virus challenge.

In addition to being tools for preventing HIV-1 infection, BnAbs are potentially valuable instruments for treating and/or curing HIV-1 infection (15, 16). Indeed, the PGT121 BnAb has been successfully used to reduce viral loads in SHIV-infected macaques (17). Assessment of SHIV-infected macaques treated with PGT121 at a dose of 10 mg/kg revealed that the Ab not only induced a decrease in plasma viremia, but also decreased the frequency of cells harboring viral DNA when used alone or in combination with 2 other BnAbs. This observation could be consistent with PGT121 using Fc-mediated functions to clear infected cells. PGT121 is currently being studied in HIV-1–infected humans (NCT03205917 and NCT02960581, clinicaltrials.gov).

To assess the importance of Fc-dependent functions of PGT121, macaques were administered WT PGT121 or a version of the Ab with greatly diminished Fc-dependent functionality (i.e., LALA PGT121) and then challenged with cell-associated SHIV$_{SF162P3}$. In a second experiment, macaques were administered an NK cell–depleting Ab prior to WT PGT121 administration and cell-associated SHIV$_{SF162P3}$ challenge. Last, a series of SHIV$_{SF162P3}$-infected macaques were administered WT or LALA PGT121 and followed up for determination of plasma viremia and viral DNA within PBMCs. The results showed that Fc-mediated functions were at least partially redundant for the protective and therapeutic capacity of the PGT121 BnAb.

## Results

*Characterization of WT and LALA PGT121 Abs.* To investigate the role of Fc-dependent functions in PGT121-conferred protection and therapeutic benefits, we studied both WT PGT121 and LALA PGT121, a version mutated to diminish FcR binding. Initially, we screened both WT and LALA PGT121 as well as WT and LALA b12 for their capacity to bind the pigtail macaque FcγRIIIa using a previously reported ELISA (18). As expected, both WT PGT121 and b12 BnAbs engaged pigtail macaque FcγRIIIa dimers, while LALA BnAbs were not bound by pigtail macaque FcγRIIIa dimers (Figure

1A). We next assessed the ability of pigtail macaque effector cells from 16 macaques to utilize WT and LALA PGT121 Abs to mediate Ab-dependent functions. In assays measuring Ab-dependent NK cell degranulation (i.e., CD107a expression) and ADCC, the WT PGT121 Ab triggered responses significantly higher than did the PGT121 LALA Ab, which induced responses similar to those seen with no Ab or HIV-1–negative intravenous IgG (IVIG) controls (Figure 1, B and C). These data highlight a markedly reduced capacity for LALA PGT121 to trigger Fc-dependent functions by macaque effector cells.

*Prevention of cell-associated virus challenge by WT and LALA PGT121.* To assess the relative abilities of WT and LALA PGT121 to protect against challenge with cell-associated SHIV, we implemented a previously described i.v. challenge model (11). In our earlier study, we demonstrated that WT PGT121 could provide partial protection (i.e., in 3 of 6 animals) against challenge with $2.45 \times 10^7$ splenocytes from a SHIV$_{SF162P3}$-infected donor animal (11). For the current experiments, we used a 5-fold-reduced challenge dose of $5 \times 10^6$ splenocytes administered i.v. We observed that the challenge dose of $5 \times 10^6$ cells readily infected 4 control animals that received 1 mg/kg human IgG1 isotype control i.v. 1 hour prior to challenge. All control animals had detectable plasma viral loads and cell-associated viral DNA 1 week after challenge (Figure 2, A and B) and developed Abs against both gp41 (1:1,000 plasma dilution) and gp120 (1:50 plasma dilution) within 2 to 3 weeks after challenge (Figure 2, C and D).

To assess whether WT PGT121 prevented infection with the challenge dose of $5 \times 10^6$ SHIV-infected splenocytes, 6 animals were infused i.v. with 1 mg/kg WT PGT121 and challenged 1 hour later. We observed that all animals receiving WT PGT121 were protected from infection. No plasma SHIV RNA or cell-associated viral DNA was detected at any of the tested time points (Figure 2, A and B), and none of the animals developed anti-gp41 Abs (1:1,000 plasma dilution, Figure 2C). It should be noted that 1 animal (FB21) in the WT PGT121 group had to be euthanized 2 weeks after the challenge for non-SHIV–related reasons. We detected anti-gp120 Abs (1:50 plasma dilution) in all animals 1 week after challenge (Figure 2D). This measurement reflects the presence
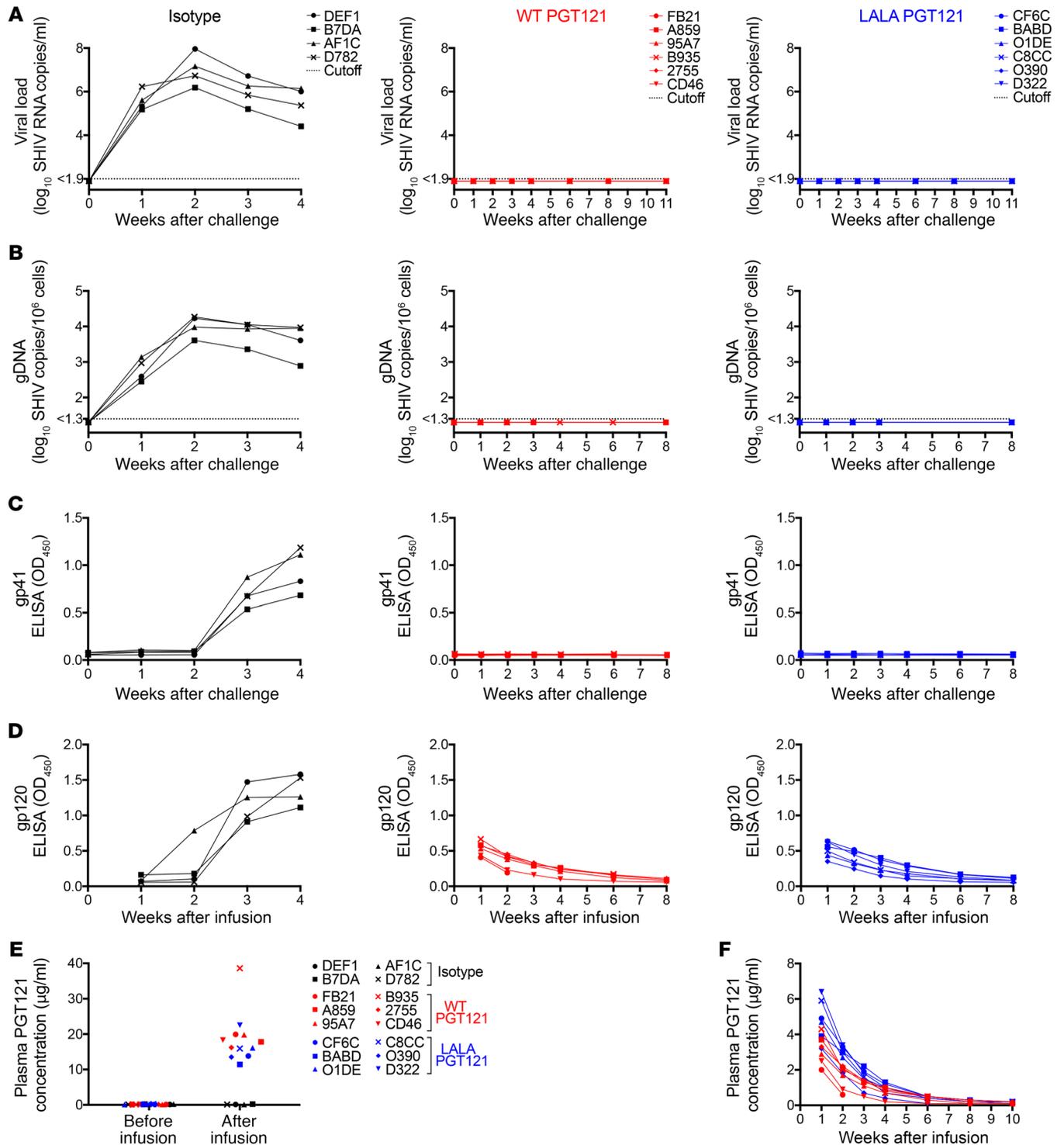
**Figure 2. Protection from cell-associated SHIV$_{SF162P3}$ challenge by WT and LALA PGT121.** Sixteen pigtail macaques were infused with 1 mg/kg isotype control Ab ($n = 4$; black; left panel), WT PGT121 ($n = 6$; red; middle panel), or LALA PGT121 ($n = 6$; blue; right panel) 1 hour before being i.v. challenged with cell-associated SHIV$_{SF162P3}$. Graphs depict (**A**) plasma viral loads and (**B**) cell-associated viral DNA of the animals in the weeks after challenge. Dotted black lines in the graphs represent the sensitivity cutoffs. (**C**) Seroconversion against HIV-1 gp41 in the weeks after challenge with cell-associated SHIV$_{SF162P3}$ was measured with an ELISA using a 1:1,000 dilution of plasma. (**D**) The presence of infused WT and LALA PGT121 Abs was assessed using an ELISA to detect gp120-specific Abs. Graphs depict the relative ODs for 1:50 dilutions of plasma samples in the weeks after infusion. The rise in OD in the animals infused with isotype control Ab reflects seroconversion against HIV-1 gp120. (**E**) Plasma concentrations of WT and LALA PGT121 before infusion and 30 minutes after infusion in all 16 animals. (**F**) Plasma PGT121 concentration over a 10-week period after infusion in the animals that received WT and LALA PGT121.

of the WT PGT121 Ab administered before the challenge. Assessment of plasma from the animals infused with WT PGT121 thirty minutes after infusion revealed a median PGT121 concentration of 19.1 μg/ml (range: 16.2–38.6 μg/ml) (Figure 2E). By 1 week after infusion, the median concentration of WT PGT121 was 3.1 μg/ml (2.0–4.3 μg/ml), and this concentration gradually waned, with a median half-life of 11.2 days (Figure 2F).

The LALA variant of the b12 BnAb was previously reported to be inferior in its capacity to protect against a cell-free SHIV challenge (2). To assess the protective capacity of LALA PGT121, six animals were concurrently i.v. infused with 1 mg/kg Ab and challenged one hour later with $5 \times 10^6$ splenocytes, as above. We found that all animals receiving LALA PGT121 were protected from infection, showing no viremia, no cell-associated viral DNA, and no gp41 seroconversion, which was identical to what we observed in animals receiving WT PGT121 (Figure 2, A–C). Thirty minutes after infusion, just prior to the challenge with cell-associated SHIV$_{SF162P3}$, the median plasma concentration of LALA PGT121 was 14.9 μg/ml (11.4–22.5 μg/ml) (Figure 2E). A comparison of the plasma concentrations of WT and LALA PGT121 Abs at the 30-minute post-infusion time point revealed slightly higher Ab concentrations in the animals infused with WT PGT121 prior to challenge (Mann-Whitney $U$ test $P = 0.04$). One week after infusion, the median plasma concentration of LALA PGT121 was 4.8 μg/ml (3.2–6.4 μg/ml), and this concentration gradually waned, with a median half-life of 9.7 days (Figure 2F).

*WT PGT121–conferred protection from cell-associated virus in the context of partial NK cell depletion.* The observation that LALA PGT121, which failed to elicit Ab-dependent effector cell functions in vitro, protected macaques from cell-associated virus challenge implies that Fc-dependent functions are not necessary for PGT121-conferred protection. Pigtail macaque NK cells are important effector cells capable of eliminating susceptible target cells through both FcγRIIIa ligation and FcR-independent stimulation (19, 20). To assess whether NK cell functions are important for achieving PGT121-conferred protection from cell-associated virus challenge, we depleted macaque FcγRIIIa⁺ NK cells using a murine anti-FcγRIIIa Ab (clone 3G8), as previously described (21, 22). Nine animals were assigned to undergo NK cell depletion and infused with 10 mg/kg anti-FcγRIIIa Ab. Two control animals were infused with saline. One day after anti-FcγRIIIa Ab infusion, the macaques had a partial reduction in NK cell frequency, with an average 61.2% reduction in peripheral blood NK cell frequency (Figure 3A). The 2 control animals concurrently infused with saline did not exhibit a decrease in peripheral blood NK cells 1 day after infusion.

To assess the impact of partial NK cell depletion on the capacity of WT PGT121 Abs to protect macaques from cell-associated virus, 1 day after anti-FcγRIIIa Ab infusion, 6 NK cell–depleted animals were infused i.v. with 1 mg/kg WT PGT121, and 3 animals were infused with 1 mg/kg human IgG1 isotype control. As a positive control for protection, the 2 macaques not depleted of NK cells were infused with 1 mg/kg WT PGT121. One hour following infusion of WT PGT121 or isotype control, all animals were challenged with cell-associated SHIV$_{SF162P3}$ ($5 \times 10^6$ splenocytes i.v., as above). The animals partially depleted of NK cells that received an isotype control Ab were readily infected, showing high levels

of both plasma SHIV RNA 1 week after challenge (Figure 3B) and cell-associated viral DNA 2 weeks after challenge (Figure 3C), and developing anti-gp41 (Figure 3D) and anti-gp120 (Figure 3E) Abs within 3 to 4 weeks of challenge. Infusion of WT PGT121 protected all animals from infection, regardless of NK cell depletion status. The 6 WT PGT121–infused NK cell–depleted animals did not exhibit viremia or cell-associated viral DNA at any time point screened (Figure 3, B and C), nor did they develop anti-gp41 Abs (Figure 3D). One week after challenge, these animals had detectable anti-gp120 plasma Abs that waned over time, confirming the presence of infused WT PGT121 Ab (Figure 3E). Assessment of plasma concentrations of WT PGT121 one week after infusion revealed a median concentration of 4.2 μg/ml (1.3–7.6 μg/ml) across the eight infused animals (Figure 3F).

*Therapeutic benefits of WT and LALA PGT121.* It has previously been reported that rapid control of persistent SHIV$_{SF162P3}$ viremia can be achieved by PGT121 infusion at 10 mg/kg (17). Given that PGT121 exhibits the capacity to trigger ADCC (11, 12), viral control might have been at least partially facilitated by the Fc-mediated killing of SHIV-infected cells. We assessed the ability of WT and LALA PGT121 at 1 mg/kg to control established SHIV infections. Twelve SHIV$_{SF162P3}$-infected macaques with persistently detectable plasma SHIV RNA and cell-associated viral DNA levels were infused with 1 mg/kg WT or LALA PGT121 and followed for viremia 24 hours, 36 hours, 48 hours, 72 hours, and 1 to 9 weeks after infusion. Two of the twelve animals received two treatments with WT or LALA PGT121, yielding fourteen treatments in total (seven each for WT and LALA). Infusion of either WT or LALA PGT121 resulted in a rapid decline in plasma viremia that eventually rebounded in most animals (Figure 4A). The rate of viremia decay within the first 72 hours was similar for both WT and LALA PGT121 infusions ($P = 0.94$, Figure 4C). Despite the fact that a decline in plasma viremia was detected in animals treated with WT or LALA PGT121, the treated animals did not exhibit marked decreases in total viral DNA in PBMCs (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI122466DS1), and no difference in DNA decay rates were noted between the animals treated with WT or LALA PGT121 ($P > 0.99$) (Figure 4, B and D). One of the SHIV$_{SF162P3}$-infected animals studied received both WT and LALA PGT121 infusions and had similar profiles of SHIV$_{SF162P3}$ RNA decline (Supplemental Figure 2). Given the large range of decay rates of plasma SHIV RNA across the animals studied, we also tested for differences in RNA and DNA decay rates between WT and LALA PGT121–infused animals using a linear mixed-effects analysis that better accounted for the variability between animals. This analysis also showed no difference in RNA or DNA decay rates between WT and LALA PGT121 treatments ($P = 0.77$ and $P = 0.81$ for RNA and DNA, respectively). Together, these data suggest that WT and LALA PGT121 Abs at a dose of 1 mg/kg conferred similar therapeutic effects in SHIV$_{SF162P3}$-infected animals.

Previous assessment of the therapeutic effect of WT PGT121 used 10 mg/kg (17), and we therefore assessed whether a marked difference occurred in terms of SHIV$_{SF162P3}$ RNA decline or PBMC SHIV DNA levels in 4 additional SHIV$_{SF162P3}$-infected pigtail macaques treated with 10 mg/kg WT or LALA PGT121 (2 animals each; orange and green lines in Supplemental Figure 3). As expected, given the higher dose, we observed a more prolonged
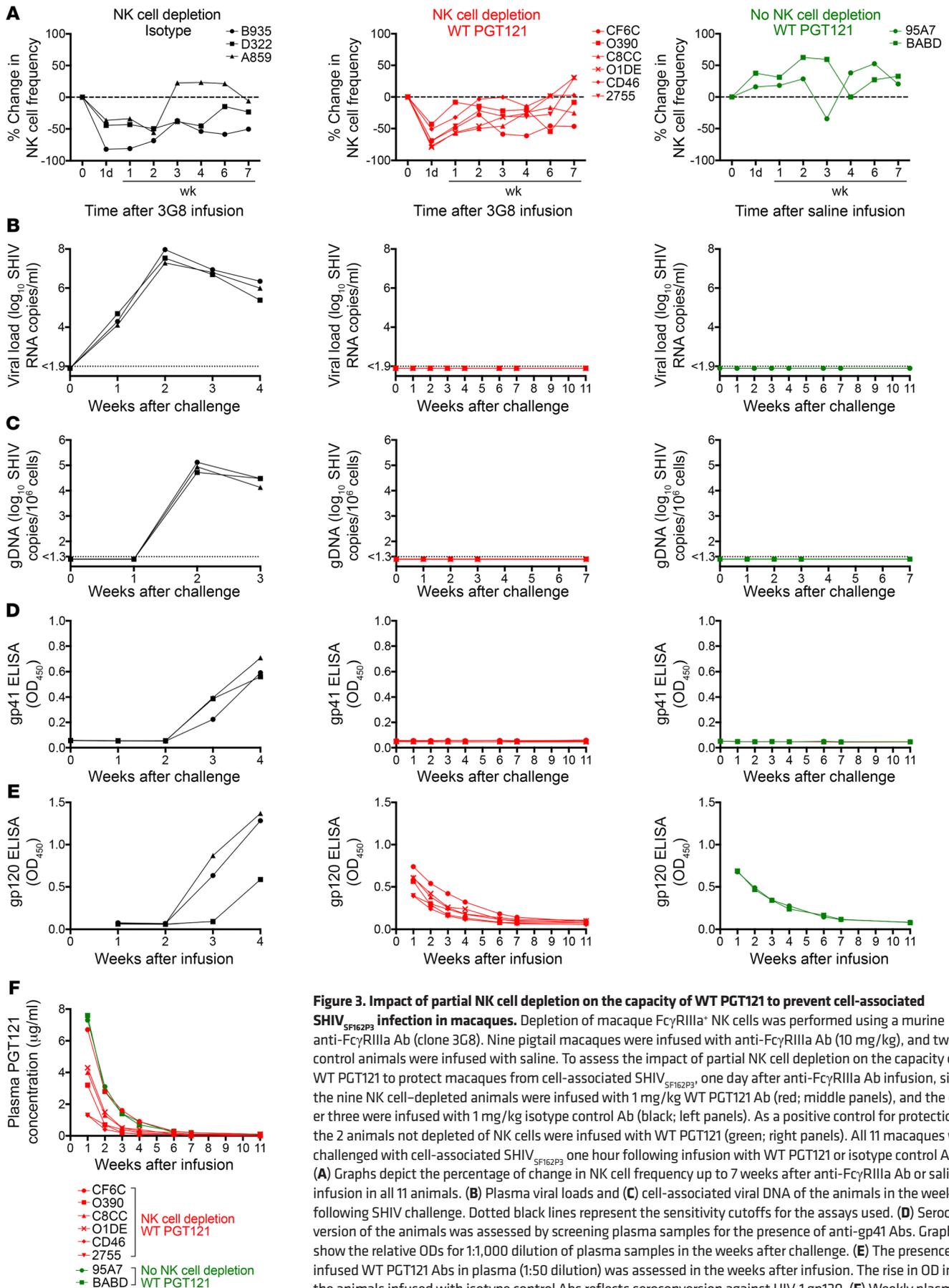
**Figure 3. Impact of partial NK cell depletion on the capacity of WT PGT121 to prevent cell-associated SHIV$_{SF162P3}$ infection in macaques.** Depletion of macaque FcγRIIIa+ NK cells was performed using a murine anti-FcγRIIIa Ab (clone 3G8). Nine pigtail macaques were infused with anti-FcγRIIIa Ab (10 mg/kg), and two control animals were infused with saline. To assess the impact of partial NK cell depletion on the capacity of WT PGT121 to protect macaques from cell-associated SHIV$_{SF162P3}$, one day after anti-FcγRIIIa Ab infusion, six of the nine NK cell–depleted animals were infused with 1 mg/kg WT PGT121 Ab (red; middle panels), and the other three were infused with 1 mg/kg isotype control Ab (black; left panels). As a positive control for protection, the 2 animals not depleted of NK cells were infused with WT PGT121 (green; right panels). All 11 macaques were challenged with cell-associated SHIV$_{SF162P3}$ one hour following infusion with WT PGT121 or isotype control Ab. (**A**) Graphs depict the percentage of change in NK cell frequency up to 7 weeks after anti-FcγRIIIa Ab or saline infusion in all 11 animals. (**B**) Plasma viral loads and (**C**) cell-associated viral DNA of the animals in the weeks following SHIV challenge. Dotted black lines represent the sensitivity cutoffs for the assays used. (**D**) Seroconversion of the animals was assessed by screening plasma samples for the presence of anti-gp41 Abs. Graphs show the relative ODs for 1:1,000 dilution of plasma samples in the weeks after challenge. (**E**) The presence of infused WT PGT121 Abs in plasma (1:50 dilution) was assessed in the weeks after infusion. The rise in OD in the animals infused with isotype control Abs reflects seroconversion against HIV-1 gp120. (**F**) Weekly plasma concentrations of PGT121 are depicted in the graph for all animals infused with WT PGT121.
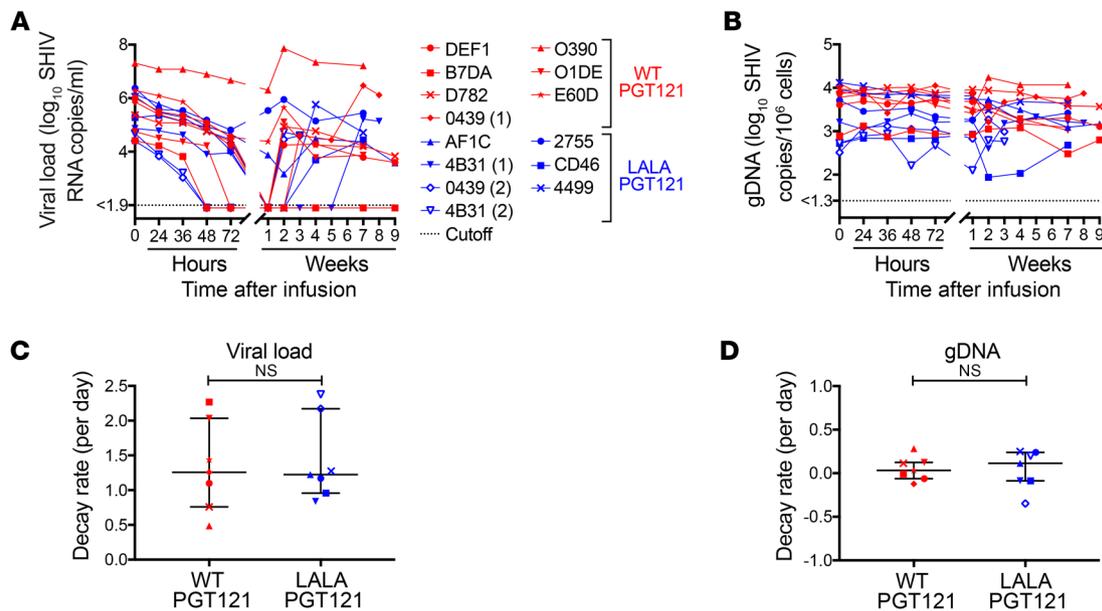
**Figure 4. Therapeutic efficacy of WT and LALA PGT121 in controlling established SHIV$_{SF162P3}$ infection.** The capacity of WT and LALA PGT121 to control established SHIV$_{SF162P3}$ infection was assessed by infusing SHIV$_{SF162P3}$-infected macaques with 1 mg/kg WT (*n* = 7; red) or LALA PGT121 (*n* = 7; blue). Graphs show (**A**) the plasma viral loads and (**B**) PBMC viral DNA of the animals in the hours and weeks after WT PGT121 or LALA PGT121 infusion. Dotted black lines represent the sensitivity cutoffs for the assays used. (**C** and **D**) Rates of decay of viral RNA and DNA in the first 72 hours across the 14 treatments with either WT or LALA PGT121. Data are depicted as the median and IQR. Decay rates were compared using Mann-Whitney *U* tests. *P* < 0.05 was considered statistically significant.

suppression of viremia, however, the rates of decline in SHIV RNA within the first 72 hours fell within the range of values observed for animals treated with the 1-mg/kg dose. The rates of PBMC SHIV DNA decay following administration of the 10-mg/kg WT or LALA PGT121 dose within the first 72 hours were generally low, with the rates for 3 animals falling within the range of the values observed for animals treated with 1 mg/kg Ab. One animal treated with the 10-mg/kg dose of WT PGT121 had a slightly higher PBMC SHIV DNA decay rate than was observed among the animals treated with 1 mg/kg of either WT or LALA PGT121.

## Discussion

Dissecting mechanisms through which Abs protect against HIV-1 will be useful for improving the design of Ab-based vaccines, passive prophylactics, and therapeutics. Despite its reduced ability to stimulate Fc-dependent functions when tested in vitro, LALA PGT121 protected macaques from a high-dose cell-associated SHIV$_{SF162P3}$ challenge as efficiently as did WT PGT121. Furthermore, WT PGT121 Ab protected macaques partially depleted of NK cells from cell-associated virus challenge, corroborating the inference from LALA-conferred prophylaxis that PGT121-conferred protection does not require Fc-dependent NK cell responses. Last, we show that both the WT and LALA PGT121 Abs were equally capable of rapidly reducing plasma viremia in SHIV$_{SF162P3}$-infected animals. Taken together, our work suggests that the Fc-mediated functions of PGT121 are not necessary for the conferral of protective and therapeutic benefits.

The data presented here are consistent with those of previous studies demonstrating the capacity of WT PGT121 to protect against SHIV$_{SF162P3}$ challenge and confer therapeutic benefits (3,

11, 17). Indeed, passive administration of WT PGT121 protects macaques from mucosal and i.v. challenge with cell-free virus (3, 11). Furthermore, our finding that BnAb reduces viremia in infected animals is consistent with data from both macaques and humanized mice (17, 23).

These data build on those from our previous study showing that WT PGT121 provided partial protection from cell-associated virus challenge (11). In the current data set, we observed a more robust protection that was conferred to all animals infused with PGT121. The observed lack of breakthrough infections was probably a factor of all animals having sufficiently high plasma levels of PGT121 Abs at the time of challenge, as well as the 5-fold lower SHIV$_{SF162P3}$ challenge dose that might have made it less likely for an occult infection to become established (11). Late breakthrough of infection despite early protection by PGT121 may be an uncommon event occurring in the setting of very high-dose SHIV exposure (24).

Additionally, our in vivo observation that PGT121 provided robust protection against the cell-associated SHIV$_{SF162P3}$ challenge is consistent with several in vitro studies showing that PGT121 neutralizes both cell-free and cell-associated virus — albeit, neutralization of cell-associated virus generally requires a higher IC$_{50}$ (10, 14, 25). The neutralization potential of an Ab is conferred through the fragment of antigen binding (Fab). While non-Fab Ab characteristics, such as distinct hinge regions of IgG subclasses, can affect viral neutralization (26), introduction of the LALA mutation does not modify the ability of an Ab to neutralize virus (2). As such, the previously reported patterns of neutralization of cell-free and cell-associated virus by WT PGT121 would be expected to be similar for the LALA PGT121. Indeed, this was indicated by the protective capacity of LALA PGT121 in this study. It is important to note

that, while in vitro data support the notion that PGT121 can neutralize cell-to-cell transmission of virus by cells infected with laboratory-adapted strains of HIV-1, incomplete neutralization of cell-to-cell transmission of virus was noted when cells infected with transmitter/founder viruses were used (14). It will be important to determine whether infections following cell-associated challenges with SHIVs expressing Envs from transmitter/founder viruses are prevented by passive immunization with PGT121.

Although our data are consistent with the literature demonstrating a role for WT BnAbs in conferring protection and therapeutic benefits, the data are in apparent contradiction with the literature highlighting a role for Fc-dependent functions (2). In macaques, the b12 BnAb partially loses protective efficacy after mutation to the LALA format. The reason (or reasons) why b12 LALA loses protective efficacy but PGT121 LALA retains protective efficacy is unknown. One possibility is that the SHIV$_{SF162P3}$ challenge virus exhibits differential abilities to escape the b12 and PGT121 BnAbs. Indeed, Barouch et al. were unable to detect PGT121 neutralization–resistant viruses in SHIV$_{SF162P3}$-infected macaques treated with PGT121 (17). Furthermore, only partial PGT121 resistance was apparent after introducing the N332A mutation — a mutation that confers complete resistance to other BnAbs with N332-dependent epitopes (i.e., PGT124 and PGT128). As such, the authors suggested that the PGT121 BnAb–SHIV$_{SF162P3}$ combination had a "high bar to resistance." Alternatively, a study of therapeutic administration of the b12 BnAb to HIV-1$_{SF162}$–infected humanized mice revealed rapid generation of neutralization escape (27). Although obtained through studies evaluating the therapeutic use of BnAbs, these data suggest that infected cells established after SHIV$_{SF162P3}$ exposure in BnAb-infused animals may be dealt with differently depending on the BnAb infused. Given that SHIV$_{SF162P3}$ inefficiently escapes PGT121, virus subsequently produced by infected cells will likely be neutralized in its cell-free state or prevented from being transmitted between cells. We speculate that a higher efficiency of escape of SHIV$_{SF162P3}$ from b12 could increase the requirement for infected cells to be rapidly eliminated by Fc-mediated mechanisms to limit the opportunity for the selection, release, and spread of neutralization-resistant viruses.

Alternatively, the differential requirement for Fc-dependent functions for b12- and PGT121-conferred protection from infection could be related to additional factors, such as the epitope specificity of the Ab. Last, it is possible that BnAbs that more potently neutralize virus are less likely to require Fc-dependent functions to protect against infection. Indeed, PGT121 neutralizes SHIV$_{SF162P3}$ more potently than does b12, exhibiting a lower IC$_{50}$ in the TZM-bl neutralization assay (0.005 μg/ml versus 0.04 μg/ml) and in a neutralization assay utilizing macaque PBMCs (0.002 μg/ml versus 0.36 μg/ml) (3).

A potential caveat of our study is the degree to which the LALA mutation of the human PGT121 BnAb completely abrogated Fc-mediated functions of macaque effector cells in vivo. However, our in vitro functional data on PBMCs from a large cohort of outbred pigtail macaques (Figure 1, B and C), the differential ability of WT and LALA PGT121 to engage with pigtail macaque FcγRIIIa dimers (Figure 1A), our data showing the protective and therapeutic benefits conferred by LALA PGT121 (Figure 2 and Figure 4, respectively), and our in vivo NK depletion experiment (Figure 3)

suggest that Fc-mediated functions are not required for PGT121 to prevent or treat SHIV$_{SF162P3}$ infection in pigtail macaques. Further studies with additional BnAbs, additional Ab mutants, and across additional nonhuman primate SHIV infection models are warranted to expand these results.

An additional component of the currently presented data that differs from previously published data (17) is that we did not observe a marked decrease in proviral SHIV DNA levels in PBMCs from animals with chronic SHIV infection receiving therapeutic WT or LALA PGT121 at a dose of either 1 mg/kg or 10 mg/kg. Previous work studying the impact of PGT121 therapy on SHIV DNA levels revealed a decline in SHIV DNA after treatment with 10 mg/kg WT PGT121, either alone or in combination with 2 other BnAbs (17). Our data highlight that the therapeutic effects of WT and LALA PGT121 were similar in terms of the impact of the Abs on viremia, but we did not observe dramatic decreases in SHIV DNA in PBMCs from either WT PGT121– or LALA PGT121–treated animals. Therefore, we are unable to draw conclusions about the relative ability of WT or LALA PGT121 to eliminate infected cells. We speculate, however, that prolonged suppression of viremia with either WT or LALA PGT121 will result in the eventual decay of PBMC DNA, as is observed after antiretroviral therapy (ART).

The observation that robust Fc-dependent functions might not be necessary for the PGT121 BnAb to protect macaques from cell-associated virus infection and confer therapeutic benefits is highly surprising in the context of previous studies demonstrating a role for Fc-dependent functions for BnAb efficacy (2, 23, 28). The data presented here highlight that not all BnAbs are created equal in terms of how they prevent infection. Defining what differentiates BnAbs that are more or less reliant on Fc-dependent functions for efficacy could be key to advancing efforts to utilize BnAbs as prophylactics and therapeutics. Highly potent BnAbs that are less reliant on Fc-dependent functions would be ideal for wide human application, as these BnAbs would be less influenced by interindividual differences that tune the functionality of FcγR-expressing effector cells (29–32).

## Methods

*Animals.* Juvenile pigtail macaques were sourced from the Monash University Animal Research Platform, the Australian National macaque breeding facility. All macaques used in the protection studies were initially naive to SHIV$_{SF162P3}$ or human Ab exposure, except for the 4 isotype control animals in the first challenge experiment (DEF1, B7DA, AF1C, D782), which had previously been protected from SHIV$_{SF162P3}$ by the WT PGT121 BnAb (11).

*WT and LALA PGT121 and isotype control Ab infusions.* WT and LALA PGT121 Abs were purchased from the Center for Antibody Development and Production (Scripps Research Institute, La Jolla, California, USA), facilitated by Diane Kubitz and Dennis Burton. The human IgG1 isotype control Ab (clone 52H5/TT1204) was provided by Keith Reimann (NIH Nonhuman Primate Reagent Resource, Mass-Biologics of the University of Massachusetts Medical School, Boston, Massachusetts, USA). The Abs were infused into macaques i.v. at 1 mg/kg 1 hour prior to i.v. challenge, as previously described (11).

For experiments assessing the impact of NK cell depletion on the ability of WT PGT121 to protect against i.v. challenge, the ani-

mals were i.v. infused with 10 mg/kg murine anti-human FcγRIIIa Ab (clone 3G8; supplied by Keith Reimann, NIH Nonhuman Primate Reagent Resource, MassBiologics of the University of Massachusetts Medical School) 1 day prior to i.v. infusion with WT PGT121 and i.v. SHIV challenge.

For experiments assessing the therapeutic value of WT and LALA PGT121 Abs, viremic animals were infused with 1 mg/kg Ab i.v. at various time points after SHIV$_{SF162P3}$ infection (range: 4–80 weeks). A subset of 4 viremic animals were infused with 10 mg/kg WT ($n = 2$) or LALA ($n = 2$) PGT121 Ab i.v. 8 weeks after SHIV$_{SF162P3}$ infection.

*Cell-associated SHIV$_{SF162P3}$ i.v. challenge.* One hour after infusion of human mAbs, the animals were challenged i.v. with $5 \times 10^6$ splenocytes derived from an allogeneic SHIV$_{SF162P3}$-infected pigtail macaque. This previously described cell-associated SHIV$_{SF162P3}$ stock was derived from splenocytes isolated from a SHIV$_{SF162P3}$-infected animal 2 weeks after infection (11). Briefly, splenocytes were isolated from homogenized splenic tissue, washed twice in RF10 media (RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin and L-glutamine; Life Technologies, Thermo Fisher Scientific), and cryopreserved in liquid nitrogen. Immediately upon thawing, the splenocytes were utilized without further intervention for i.v. challenge.

The $5 \times 10^6$ splenocyte dose contained $2 \times 10^6$ copies of cell-associated SHIV RNA and $1.5 \times 10^4$ copies of cell-associated SHIV DNA (11). Using cumulative challenge data from the current study and a previously reported limited in vivo titration of the cell-associated SHIV$_{SF162P3}$ stock, we identified the dose of $5 \times 10^6$ splenocytes used in this study as corresponding to approximately 462 animal infectious doses (95% CI: 53–4,054), using a modified limiting dilution analysis (33).

*Generation of pigtail macaque FcγRIIIa dimer and ELISA to assess FcγRIIIa engagement of Abs.* A dimeric FcγRIIIa expression vector was constructed using a codon-optimized synthetic (GeneArt, Invitrogen, Thermo Fisher Scientific) *Macaca nemestrina* sequence (NCBI Reference Sequence [RefSeq] Database: XP_011768496) and produced in Expi293F cells (Thermo Fisher Scientific) expressing ER-localized BirA ligase, as previously described (18). The hexahistidine- and biotin-tagged protein was purified using TALON Superflow (BD Biosciences) and Superose 6 chromatography (GE Lifesciences).

ELISA plates (Nunc) were coated with 10 μg/ml AffiniPure goat F(ab′)$_2$ specific for human IgG-F(ab′)$_2$ (Jackson ImmunoResearch) and used to capture the indicated concentrations of WT b12, PGT121, and their respective LALA mutants (gifts from Dennis Burton, The Scripps Institute, La Jolla, California, USA). The binding of *M. nemestrina* biotinylated dimeric rsFcγRIIIa was detected as previously described (18).

*Ab-dependent NK cell activation assay.* Activation of macaque NK cells by antigen-bound PGT121 was measured using a modified version of a previously described plate-bound Ab-dependent NK cell activation assay (11, 34). Briefly, 96-well ELISA plates (Nunc) were coated with 600 ng/well of HIV-1$_{SF162}$ gp140 (obtained from Leo Stamatatos through the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases [NIAID]) overnight at 4°C. The wells were blocked with PBS and 5% BSA for 1 hour at 37°C and then incubated for 2 hours at 37°C in the absence of Ab or presence of 20 μg/ml WT PGT121 or LALA PGT121 Ab. Next, $1 \times 10^6$ freshly isolated macaque PBMCs were added to each well and incubated at 37°C for 5 hours in the presence of anti-human CD107a-allophycocyanin-H7 (clone H4A3; BD Biosciences), 2 μg/well brefeldin A (Sigma-Aldrich), and 5 μg/ml monensin (GolgiStop; BD Biosciences). Cells were then

incubated with anti-human CD8–peridinin chlorophyll protein complex (clone SK1; BD Biosciences), anti-human CD3–Pacific Blue (clone SP34-2; BD Biosciences), and anti-human NKG2A-allophycocyanin (clone Z199; Beckman Coulter) Abs and 1 mM EDTA for 30 minutes at room temperature in the dark. Cells were fixed with 1% formaldehyde and acquired on a LSRFortessa Flow Cytometer (BD Biosciences). Analysis was performed using FlowJo software, version 10.0.8.

*Infected cell elimination ADCC assay.* A modified version of a previously described flow cytometry–based infected cell elimination assay was performed to assess ADCC mediated by WT or LALA PGT121 by macaque effector cells (11, 35, 36). Briefly, the HIV-1$_{LAV}$–infected 8E5/LAV cell line (obtained from Thomas Folks through the NIH AIDS Reagent Program, Division of AIDS, NIAID) was first stained with LIVE/DEAD Near-IR Viability Dye (Life Technologies, Thermo Fisher Scientific) and the cell proliferation dye eFluor 670 (eBioscience). Freshly isolated macaque PBMCs were stained with eFluor 450 (eBioscience). Effector macaque PBMCs ($10^6$ cells) were incubated with target 8E5/LAV cells ($2 \times 10^5$) at a 5:1 ratio in the presence of human IVIG (20 μg/ml) or WT or LALA PGT121 (20 μg/ml) Abs for 5 hours at 37°C with 5% $CO_2$. Cells were then fixed with 1% formaldehyde, permeabilized with 1× FACS permeabilization buffer (BD Biosciences), and stained with a phycoerythrin-conjugated Ab against HIV-1 p24 (clone KC57, Beckman Coulter) to detect HIV-1–infected target cells. Cells were finally fixed with 1% formaldehyde and acquired on a LSRFortessa Flow Cytometer (BD Biosciences). ADCC was calculated using the following formula: percentage of p24$^+$ cells in ([targets + effectors] – [targets + effectors + Ab])/[targets only] × 100.

*Seroconversion to gp41 and gp120.* ELISAs were performed using a previously reported protocol (11) to assess for Abs against gp41 (ELISA detects seroconversion but does not detect the infused PGT121 Ab) and detect Abs against gp120 (ELISA detects both seroconversion and the infused PGT121 Ab).

*Assessment of SHIV RNA and DNA.* Quantification of the viral RNA copies within plasma and cell-associated viral DNA within PBMCs was accomplished by droplet digital PCR (ddPCR) using a TaqMan probe (6-FAM, MGBNFQ; Thermo Fisher Scientific) designed to recognize a conserved region within SIV *Gag* (the primers and probe are listed in Supplemental Table 1). Briefly, viral RNA was isolated from 140 μl plasma, and 10 μl of the viral RNA was subjected to reverse transcription using SuperScript III (Invitrogen, Thermo Fisher Scientific), according to the manufacturer recommendations. To normalize plasma samples and the efficiencies of reverse transcription and PCR, all viral RNA samples were spiked with a known concentration of an in vitro–transcribed importin 8 (*IPO8*) reference RNA, which was further detected in a multiplexed reaction with SHIV by using an *IPO8* probe (HEX, BHQ1).

The ddPCR reaction consisted of 1× ddPCR supermix for probes (no dUTP, Bio-Rad), 1x*IPO8* primers-probes (dHsaCPE5044719, Bio-Rad), 1 μM forward and reverse SIV Gag primers, 200 nM probe, and 6 μl cDNA in a 24-μl reaction. Following the generation of droplets (15,000–18,000 on average), thermal cycling was conducted as follows: 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds, and 60°C for 1 minute, followed by 98°C for 10 minutes (ramp rate 2°C/s for each step) on a C1000 Touch Thermal Cycler (Bio-Rad). The droplets were subsequently read on a QX200 Droplet Reader (Bio-Rad), and data were analyzed with QuantaSoft 1.7.4 software. The positive droplets were designated on the basis of the minus reverse transcriptase control (–RT), the no-template controls (NTCs), and fluorescence minus one

(FMO) controls (SIV⁻/IPO8⁺, SIV⁺/IPO8⁻, and SIV⁺/IPO8⁺) included in each run. Each sample was run in duplicate, and the merged data (copies/μl) were used to determine the amount of $SHIV_{SF162P3}$ RNA/ml plasma. To probe the limits of the protection observed, a subset of PBMC samples was also assessed for SHIV DNA in a ddPCR assay with the same primers-probe sets used for SHIV RNA. Briefly, genomic DNA (gDNA) was extracted from PBMCs using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's protocol. Equal amounts of gDNA (100 ng) were consequently used to determine the amount of cellular SHIV DNA per $10^6$ PBMCs. The limit of detection (LOD) for SHIV was determined using a series of 10-fold dilutions of the known concentration of RNA and DNA standards, and the results were plotted as expected versus the measured copy number. The LOD of 1.90 $\log_{10}$ copies RNA/ml plasma and 1.3 $\log_{10}$ SHIV DNA copies/$10^6$ cells was calculated according to the endpoint of the standard curve where it was no longer linear.

*Statistics.* The decay rates of Ab, viral RNA, and cellular DNA for each animal were estimated using an ordinary linear regression method on the log-transformed values of the measurements. The decay values were compared using 2-tailed Mann-Whitney *U* tests. All analyses were performed using GraphPad Prism software, version 7. To confirm and validate that no differences in the rates of decay of viral RNA and cellular DNA occurred between WT and LALA PGT121–treated animals, a linear mixed-effects regression analysis was also performed (*nlme* library, version 3.1-131, in R statistical software, version 3.3.3). Additional reported analyses were conducted using GraphPad Prism software, version 7. Paired data were compared using 2-tailed Wilcoxon matched-pairs tests. In scenarios involving more than 2 groups, the Freidman test with Dunn's post hoc tests was applied. Nonpaired data were compared using 2-tailed Mann-Whitney *U* tests. For all statistical tests, *P* values of less than 0.05 were considered significant .

*Study approval.* The Australian Commonwealth Scientific and Industrial Research Organization Animal Health Animal Ethics Committee approved all macaque studies.

## Author contributions

MSP and SJK designed the study, conducted experiments, analyzed data, and prepared the manuscript. WSL, ABK, TA, GK, AKW, BW, and PMH conducted experiments, contributed to data analyses, and revised the manuscript. MPD and AR assisted with statistical analyses.

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