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Introduction
Since the discovery of human immunodeficiency virus type 1 (HIV-1) as the causative agent of AIDS in 1983, the search for an effective vaccine or a therapeutic cure has been the top priority in the fight against the expanding HIV/AIDS pandemic. However, because of the tremendous difficulties of HIV-1 vaccine design, generating an appropriate immunogen to elicit broadly neutralizing antibodies (bnAbs) against genetically divergent HIV-1 subtypes (1, 2) has been unsuccessful. With the recent discovery of numerous antibodies (bnAbs) against genetically divergent HIV-1 subtypes (1, 2), it has become evident that viral coevolution is likely required to drive B cell maturation to induce potent bnAbs during the natural course of infection (2, 10, 11). While there has been an increase in efforts to identify structure-guided novel immunogen design for an efficacious vaccine (3, 12–14), using existing bnAbs as passive immunization is an alternative approach for HIV-1 prophylaxis and immunotherapy (4, 7, 15–20).

Numerous studies have investigated the potency, breadth, crystal structure, and mode of action of selected bnAbs, including their combined use both in vitro and in vivo (16, 21–23). Naturally occurring resistant viruses, however, are readily found against these bnAbs when tested individually (9, 21). The bnAb-based monotherapy failed to induce durable suppression of plasma viremia as resistant viruses emerged (20, 24). To improve HIV-1 neutralization breadth and potency, bispecific bnAbs (bs-bnAbs) have been engineered using the available gene sequences of bnAbs (25–29). In particular, by CrossMAb and knobs-into-holes technologies, bs-bnAb 10E8V2.0/iMab displays exquisite HIV-1-neutralization activity in humanized mouse models of HIV-1 prevention and treatment (30). Although bs-bnAbs are promising, their clinical development faces large-scale manufacturing challenges and concerns of possible immunogenicity and poor pharmacokinetic properties. Gene transfer of bs-bnAbs may also face several technical challenges. For example, bs-bnAbs generated by the knobs-into-holes method require codelivery of 2 or more genes into the same cell for proportional expression and assembly of antibody light and heavy chains (30). Nevertheless, the recent FDA approval of a CD19- and CD3-targeting bispecific antibody for acute B
cell lymphoblastic leukemia has shed light for bs-bnAb-based immunotherapy (31); allowing this bi-specific antibody to be used for clinical development.

To date, the immunotherapeutic potential of gene-transferred bs-bnAbs has not been investigated in vivo against HIV-1 infection.

In this study, we developed a single gene–encoded tandem bispecific immunoadhexit molecule (BiIA), namely BiIA-SG. Engineered immunoadhexit (IA) is an antibody-like molecule, and in this study, IA refers to such molecules that contain the antigen-binding domain of the single-chain variable fragment (scFv) of bnAbs in fusion with the immunoglobulin constant region, including the hinge and Fc fragment (e.g., IgG-Fc) but without the constant light chain (CL)/constant heavy chain 1 (CH1) (32, 33). We show that BiIA-SG not only displays a potent average IC_{50} value of 0.073 μg/ml against all 3 panels of 124 genetically divergent HIV-1 strains tested, but also completely prevents diverse live viral challenges in humanized mice. Mechanistically, the improved breadth and potency of the engineered BiIA-SG are associated with the preservation of 2 scFv binding domains of each parental bnAb, which is different from the conventional knobs-into-holes bs-bnAbs. Importantly, gene transfer of BiIA-SG displays the promising activity of eliminating HIV-1–infected cells in many humanized mice. Herein, we provide a proof-of-concept that BiIA-SG is a promising agent for bs-bnAb–based postexposure viremia control and immunotherapy against HIV-1 infection.

Results

**Engineering of a single gene–encoded tandem BiIA-SG.** Before engineering BiIAs, we synthesized codon-optimized scFvs of bnAbs including PG9, PG16, PGT128, VRC01, and Hu5A8 (7–9). The variable light chain (VL)/variable heavy chain (VH) domain of each scFv was engineered as a corresponding IA by fusion with human IgG1-Fc to generate IA-PG9, IA-PG16, IA-PGT128, IA-VRC01, and IA-Hu5A8 (7–9). The expression of released soluble IAs was readily detected by Western blot after transient transfection of human 293T cells (Figure 1C). While all IAs exhibited specific anti–HIV-1ADA activity, only IA-PGT128 displayed similar potency and the same sigmoidal slope of 100% neutralization as previously described for the native PGT128 (34). Furthermore, we measured the anti–HIV-1 activity of combined IAs in checkerboard experiments (35, 36). We found that IA-PGT128 in combination with IA-Hu5A8 exhibited the best synergistic effect based on computational synergy volumes (Figure 1E and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI96764DS1). We therefore focused on IA-PGT128 and IA-Hu5A8 for BiIA construction. For mechanism study, we constructed BiIAs by 2 methods. Using the knobs-into-holes method, we generated a double gene–encoded (DG) bispecific IA (BiIA-DG) with the modified IgG1-Fc domain, as previously described (Figure 2A) (25, 37). Using a gene tandem fusion method, we also constructed a single gene–encoded (SG) BiIA (BiIA-SG) through fusion of the PGT128 VH/VH to the N-terminal of IA-Hu5A8 VL/VH in tandem (Figure 2B). As a result, BiIA-SG is structurally unique with 4 scFv binding domains (2 for HIV-1 gp120 and 2 for CD4) as compared with BiIA-DG or other knobs-into-holes bs-bnAbs that contain 2 scFv binding domains (1 for each of the 2 target antigens), as previously described by others (25, 37).

As compared with parental IA-PGT128 and IA-Hu5A8, the expression of BiIA-SG and BiIA-DG was readily detected from transfected 293T cell supernatants with expected sizes determined by Western blot (Figure 2C). To determine their dual specificity, we measured protein binding by 2 methods, ELISA and...
BiIA-SG displays significantly enhanced potency and breadth against HIV-1. To compare the breadth and potency of BiIA-SG and BiIA-DG with parental IA-PGT128 and IA-Hu5A8, we first tested our University of Hong Kong (HKU) panel of 40 pseudoviruses (Supplemental Figure 3), which covered major global HIV-1 subtypes B/B’, CRF01_AE, and C/CRF07_BC/CRF08_BC (34, 38). These 40 pseudoviruses included 8 acute and transmitted/founder (T/F) strains of various subtypes. VRC01-IgG, one of the second-generation bnAbs in clinical trials (39), was also included for comparison. Since the molecular weights of IAs are smaller than those of BiIAs and regular antibodies, equimolar concentration (nM) is used for comparison. We found that the mean IC_{50} and IC_{90} values of BiIA-SG (1.1 nM/17.9 nM) were significantly better than those of BiIA-DG (35.1 nM/85.3 nM), IA-PGT128 (69.0 nM/99.6 nM), IA-Hu5A8 (13.0 nM/73.4 nM), and VRC01-IgG (29.2 nM/70.0 nM) (Figure 3A). As a control, the knobs-into-holes BiIA-DG was not significantly better than parental IA-PGT128 and IA-Hu5A8. This finding is consistent with the bs-bnAb PGT128/iMab generated by the knobs-into-holes and the CrossMAb technologies (30). In contrast, BiIA-SG was able to neutralize 65% (26/40) of pseudoviruses with IC_{50} values less than 1 nM (~0.15 μg/ml). Moreover, 13 IA-PGT128–resistant, 8 IA-Hu5A8–resistant, and 2 dual-resistant viruses (Figure 3B) as well as 5 VRC01-resistant viruses (Supplemental Figure 3) were neutralized by BiIA-SG with improved IC_{90} or IC_{50} values. The improved potency of BiIA-SG was also observed with live replicating HIV-1 strains (Figure 3C). To further investigate its antiretroviral potency, we compared BiIA-SG with a panel of 7 bnAbs (VRC01, 3BNC117, PG09, PG16, 10-1074, PGT121, and 10E8) obtained from the NIH AIDS Reagent Program. BiIA-SG consistently exhibited the strongest potency and breadth against 8 genetically divergent and acute HIV-1 pseudoviruses, with average IC_{50} and IC_{90} values of 0.023 μg/ml and 0.107 μg/ml, respectively (Figure 4A). BiIA-SG also had similar average IC_{50} and IC_{90} values of 0.018 μg/ml and 0.111 μg/ml, respectively, against the NIH global panel of 12 HIV-1 pseudoviruses (Figure 4B) (40). In addition, BiIA-SG has been independently tested in a collaborative laboratory with average IC_{50} and IC_{90} values of 0.05 μg/ml and 0.144 μg/ml, respectively.
IC₅₀ value of 1.32 nM than that of IA-PGT128 (2.36 nM) (Figure 3C, right). Their equimolar combination achieved an IC₅₀ value of 0.79 nM that was still less potent than that of BiIA-SG by 3-fold (0.26 nM). Similar results were confirmed using the NIH global panel of 8 of 12 HIV-1 pseudoviruses based on IC₅₀ and IC₉₀ values (Figure 4B). Overall, BiIA-SG is more potent against both live and genetically divergent viral strains as compared with IA-PGT128, IA-Hu5A8, or their combination in equimolar concentration.

Mechanistically, the enhancement of BiIA-SG is probably related to higher affinity cross-linking interactions with HIV-1 gp120 based on the structural modeling (Supplemental Figure 5) (42).

BiIA-SG confers sterile protection against genetically divergent HIV-1 challenges in humanized mice. To determine the protection efficacy in vivo, we examined the effect of a single injection of BiIA-SG in the HIV-1/NSG-HuPBL model (Figure 5A) (41). A bioreactor product of BiIA-SG generated a peak of 90% purity by size exclusion chromatography analysis (Figure 5B). We then measured the pharmacokinetics and bioavailability of BiIA-SG, which was purified from Chinese hamster ovary (CHO) cells, in 4 healthy NSG-HuPBL mice (2 male [M] and 2 female [F]) and found that its calculated peripheral half-life (t₁/₂) was approximately 3.1 days (Figure 5C), which fell between the t₁/₂ values of 5.6 days for Hu5A8 and 2.2 days for PGT128 (7, 16). BiIA-SG ly, against a Tsinghua University panel of 72 HIV-1 pseudoviruses (Supplemental Figure 4). In this experiment, 21% (15/72) of pseudoviruses had IC₅₀ values less than 0.001 μg/ml. Based on all 3 panels of 124 HIV-1 pseudoviruses tested, our results demonstrate that BiIA-SG is superior to many bnAbs, including BiIA-DG, with a substantially enhanced average IC₅₀ value of 0.073 μg/ml (range < 0.001–1.03 μg/ml) and 100% breadth.

To prepare for in vivo efficacy experiments, the anti–HIV-1 activity of BiIA-SG was evaluated using 2 authentic live viral strains, the R5-tropic HIV-1JR-FL (subtype B) and the R5-tropic T/F virus HIV-1BJZS7 (subtype CRF01_AE). Both HIV-1JR-FL, a tier-2 virus relatively resistant to antibody neutralization (8), and HIV-1BJZS7, established robust systemic and mucosal infections in NOD.Cg-Prkdcscp1Il2rgtm1WjlSzJ–human peripheral lymphocyte (NSG-HuPBL) mice, as we recently described (41). We found that live HIV-1JR-FL was resistant to IA-Hu5A8 neutralization but was sensitive to IA-PGT128 with an IC₅₀ value of 12.07 nM (Figure 3C, left). The combined equimolar IA-Hu5A8 and IA-PGT128 displayed enhanced neutralizing activity with the IC₅₀ value improved by 6.4-fold to 1.88 nM compared with IA-PGT128. This improved activity, however, was still 6.2-fold less potent than BiIA-SG, which had an IC₅₀ value of 0.30 nM. The neutralization of HIV-1BJZS7 was different because IA-Hu5A8 had a slightly better IC₅₀ value of 1.32 nM than that of IA-PGT128 (2.36 nM) (Figure 3C, right). Their equimolar combination achieved an IC₅₀ value of 0.79 nM that was still less potent than that of BiIA-SG by 3-fold (0.26 nM). Similar results were confirmed using the NIH global panel of 8 of 12 HIV-1 pseudoviruses based on IC₅₀ and IC₉₀ values (Figure 4B). Overall, BiIA-SG is more potent against both live and genetically divergent viral strains as compared with IA-PGT128, IA-Hu5A8, or their combination in equimolar concentration. Mechanistically, the enhancement of BiIA-SG is probably related to higher affinity cross-linking interactions with HIV-1 gp120 based on the structural modeling (Supplemental Figure 5) (42).

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was then evaluated against HIV-1 JR-FL in preexposure prophylaxis (PrEP) experiments using a previously reported dosage (10 mg/kg, \( n = 5, 2 \text{ M}, 3 \text{ F} \)) (43, 44). Groups of NSG-HuPBL mice were injected with either BiIA-SG or a placebo (PBS) 1 hour before intraperitoneal (i.p.) inoculation with 10 ng P24 of HIV-1 JR-FL (41). All placebo mice (\( n = 5, 3 \text{ M}, 2 \text{ F} \)) were infected with peak plasma viral loads up to 10^7 copies/ml (Figure 5D), and exhibited P24 antigenemia (Figure 5E), a trend of CD4+ T cell loss over time (Figure 5F), and P24+ T cells in blood and spleens (Figures 5, G and H) at 2 weeks postinfection (wpi). In contrast, no signs of infection or CD4+ T cell loss were found in 5 of 5 mice that were pretreated with BiIA-SG. Since CRF01_AE is one of the most predominant HIV-1 sexually transmitted subtypes (45), we subsequently determined the efficacy of BiIA-SG against live HIV-1BJZS7. Using the same dose and method, we found that BiIA-SG also conferred complete protection against HIV-1 BJZS7 (Figure 5, \( n = 4, 2 \text{ M}, 2 \text{ F} \)). Moreover, all protected animals had undetectable P24+ T cells in their spleens, lungs, livers, kidneys, stomachs, small intestines, large intestines, and brains by immunofluorescence staining (Supplemental Figure 6). BiIA-SG could reach the intestinal mucosal sites within 24 hours after a single i.p. injection (Supplemental Figure 7). Our findings demonstrate that soluble BiIA-SG is effective for PrEP against genetically divergent live HIV-1 strains in humanized mice.

**Figure 4. Comparison of neutralizing activity between BiIA-SG and bnAbs against 8 acute and 12 NIH global panel HIV-1 pseudoviruses. (A) Acute HIV-1 pseudoviruses. (B) NIH global panel HIV-1 pseudoviruses. Data represent mean IC_{50} and IC_{90} values tested in duplicate. All experiments were repeated twice.**
Single injection of AAV vectored BiIA-SG eliminates HIV-infected splenocytes in humanized mice. To determine the therapeutic effect of BiIA-SG in vivo, we first compared it with cART in humanized mice. Four days after i.p. challenge with 10 ng P24 of HIV-1, mice were treated with cART (TDF+3TC+RAL regimen, daily), BiIA-SG (400 μg, every 4 days), cART+BiIA-SG combination, or placebo for 3 weeks (Supplemental Figure 8). When compared with the placebo mice that exhibited high levels of viremia starting from day 4 after viral challenge, all 3 groups of treated mice showed viral suppression with subsequent viral rebound after treatment cessation. Interestingly, the cART+BiIA-SG group showed a relatively delayed viral rebound. These results suggest that short-term treatment (3 weeks) by cART or BiIA-SG or cART+BiIA-SG is insufficient for sustained viral load control or elimination of HIV-1 infection in humanized mice.

We then sought to provide prolonged in vivo BiIA-SG expression using AAV transduction as a monotherapy (15, 46, 47). BiIA-SG derived from AAV-BiIA-SG–injected mice tends to have an estimated peripheral t1/2 of about 21.7 days (Supplemental Figure 9), which is longer than the t1/2 of 3.1 days of the product purified from CHO cells in vitro (Figure 5C). Two weeks after live HIV-1 infection, groups of NSG-HuPBL mice in 2 separate experiments were treated with a control AAV-GFP (4 × 10⁹ genomic copies [gc] n = 5, 3 M, 2 F) or with a single injection of AAV–BiIA-SG in low-, middle-, or high-dose levels (low dose, 1 × 10⁹ gc, n = 2, 1 M, 1 F; middle dose, 4 × 10⁹ gc, n = 5, 3 M, 2 F; and high dose, 1 × 10¹⁰ gc, n = 8, 4 M, 4 F). A group of 4 uninfected mice (2 M, 2 F) served as a negative control (Figure 6A). Infected animals treated with AAV-GFP or low-dose AAV–BiIA-SG exhibited persistent viremia (Figure 6B). Of note, a trend of reduced viral load over time was detected in these mice and it was probably caused by the gradual loss of human CD4+ T cells and corresponding virus-producing cells. In contrast, with increased blood concentrations of BiIA-SG over time, some infected mice treated with middle (4/5) or high doses (5/8) of AAV-
Figure 6. Therapeutic efficacy of AAV-delivered BiIA-SG in infected NSG-HuPBL mice. (A) Experimental schedule of AAV-delivered BiIA-SG for immunotherapy of HIV-1JR-FL–challenged humanized mice. A single intramuscular injection of each AAV was performed 2 weeks after HIV-1JR-FL challenge. (B) The top panel shows the peripheral viral loads tested over time among 4 groups of animals, including AAV-GFP (4 × 10^9 gc, n = 5), low-dose AAV-BiIA-SG/L (1 × 10^9 gc, n = 2), middle-dose AAV-BiIA-SG/M (4 × 10^9 gc, n = 5), and high-dose AAV-BiIA-SG/H (1 × 10^11 gc, n = 8). The bottom panel shows peripheral concentration of BiIA-SG expressed over time among the same 4 groups of animals. Each line represents data from 1 mouse. (C) The correlation between plasma viral load and BiIA-SG concentration at 11 wpi among 3 AAV–BiIA-SG groups of animals. Correlation analyses were performed by linear regression using the GraphPad Prism 5.01 program. (D) Proviral loads of peripheral T cells at 11 wpi among uninfected (n = 4) and 4 groups of treated animals by digital PCR. The y axis represents the amount of DNA copies per cell, plotted as the ratio of HIV-1 DNA to CCR5 copies for each mouse. (E) Before cell adoptive transfer at 11 wpi, donor splenocytes from uninfected and 3 groups of AAV-treated mice were tested by VOA. The y axis indicates viral load copies per milliliter culture supernatants. (F–H) Four weeks after cell adoptive transfer, plasma viral load, the frequency of peripheral P24+ T cells, and the frequency of splenic P24+ T cells were determined in individual recipient mice. Uninfected, n = 4; AAV-GFP, n = 5; AAV-BiIA-SG/M, n = 5; AAV-BiIA-SG/H, n = 8. The color-coded symbols correspond to donor and recipient relationship. (D–H) Data represent mean ± SEM; 2-tailed, unpaired, Student’s t tests were performed. ***P < 0.001; **P < 0.01; *P < 0.05.
BiIA-SG had viral loads suppressed to undetectable levels from 5–11 wpi (Figure 6B). The level of peripheral BiIA-SG was inversely correlated with the viral load at 11 wpi (Figure 6C). At this endpoint, most mice with an undetectable viral load had neither measurable proviral DNA as detected by digital PCR (Figure 6D) nor P24+ T cells in the blood or spleens (Figure 6, F–H). In contrast, live HIV-1 and HIV-infected cells were readily detected in all humanized mice receiving spleenocytes from the AAV-GFP group. Our results demonstrate that gene transfer of engineered BiIA-SG leads to the elimination of infected spleenocytes, which is likely associated with relatively prolonged in vivo expression of BiIA-SG and VOA negativity in humanized mice.

Discussion

In this study, we report to our knowledge the first single gene-encoded tandem BiIA-SG to facilitate gene transfer of a bs-bnAb-based intervention for HIV-1 prevention and immunotherapy. Each BiIA-SG molecule has 4 scFv-binding domains in total, 2 from each of its parental IAs, PGT128 and Hu5A8. It is structurally different from the previously reported bs-bnAb PGT128/iMab that contains only 1 PGT128 and 1 Hu5A8 scFv-binding domain (30). Mechanically, including 2 PGT128 scFv-binding domains in each BiIA-SG molecule is likely necessary to perform the high-affinity cross-linking interaction with HIV-1 gp120 (42). This notion is in line with the critical topology of PGT128 interaction with the gp120 outer domain at the top surface of the spike (42, 48, 49). In support of this notion, we consistently demonstrated that BiIA-SG is significantly superior to BiIA-DG (Figure 3A), which mimics the PGT128/iMab, for both improved binding to gp120 and enhanced anti-HIV-1 activity. Considering that PGT128 is among the few bnAbs that can achieve a sigmoidal slope of 100% neutralization against HIV-1 infection (34), we believe that the preservation of 2 scFv-binding domains of PGT128 in each BiIA-SG molecule is at least one of the essential mechanisms underlying its substantially improved potency and breadth.

With substantially improved breadth and potency, BiIA-SG is a promising bs-bnAb for potential clinical development. Viral genetic diversity is one of the major obstacles to bnAb-based HIV-1 prevention and immunotherapy. On the one hand, multiple HIV-1 subtypes are readily diagnosed nowadays not only in individual cities in the world (45) but also in small local communities and subpopulations, such as men who have sex with men (48, 49). On the other hand, most bnAbs are not effective against all circulating global HIV-1 strains (Figure 4). For example, PGT128 neutralized 27% and 72% of viruses examined in 2 previous studies (9, 21). In our study, PGT128 was able to neutralize 72.5% of viruses tested. Moreover, 7% of our viruses, including the live HIV-1JR-FL, were not susceptible to Hu5A8 neutralization. In contrast to either PGT128 or Hu5A8 alone, BiIA-SG not only neutralizes 100% of the 3 panels of 124 pseudoviruses tested, including the global panel of 12 HIV-1 strains, with substantially improved IC50 values, but also is more potent than many newly discovered bnAbs tested (Figure 4).

Critically, our data also demonstrate that BiIA-SG provides complete protection against live challenges with genetically divergent HIV-1JR-FL and HIV-1JR-FLA. Using the same experimental design and dosage, a previous study showed that bnAb b12 provided 50% protection against HIV-1JR-FLA in humanized mice (44). It is feasible that BiIA-SG can be applied to fight the evolving AIDS epidemic with genetically diverse viruses without the need for prior viral neutralization susceptibility tests. It may indeed offer cost effectiveness compared with the combined use of a cocktail of bnAbs (18, 22).

Patients with HIV-1 require lifelong cART mainly because the virus persists in the latent viral reservoir. One of the major tasks in HIV-1 cure research is to discover means to eliminate infected cells, especially those in the reservoir (50). Using HIV-infected humanized mice as a model, a recent study indicated that viral latency is established prior to peak viremia because cART initiated as early as 5 days after infection failed to prevent viral rebound after the cessation of the cART and bnAbs (22). Encouragingly, combination of cART and latency-reversing agents with individual or a cocktail of bnAbs has significantly decreased the latent reservoir as measured by delayed viral rebound in humanized mice (18, 22, 51, 52). It has been suggested that potent bnAbs might have the advantage to either decrease the half-lives of HIV-1-infected cells in vivo by a FcγR-dependent mechanism or enhance host humoral immunity against HIV-1 probably by forming immune complex for eliciting CD8+ T cell responses (51, 52). Moreover, by conjugating particular dual or ternary bnAbs, the recently developed bispecific 10E8v2.0/iMab and trispecific VRC01/PGDM1400/10E8 achieved exquisite breadth and potency with enhanced protective and therapeutic effects (30, 53). In addition, the hinge-modified bs-bnAb 3BNC117/PGT135 showed an improved Fab flexibility and neutralization activity (54). In our humanized mouse model, short-term treatment (3 weeks) with either cART or BiIA-SG alone, or with combined cART and BiIA-SG, was insufficient to prevent viral rebound when provided as early as 4 days after live HIV-1 infection. In contrast, a single intramuscular injection of AAV-BiIA-SG at a dose of 4 × 109 gc (middle) or 1 × 1011 gc (high) in 2 separate experiments could produce a sufficient level of functional BiIA-SG in vivo to achieve complete viral load suppression in 3 and 5 animals at 11 wpi, respectively. By 11 wpi, 5 of these 8 controller mice had neither detectable HIV-1 DNA nor any replication-competent viruses in their spleenocytes as detected by VOA and cell adoptive transfer experiments. Therefore, in vivo gene transfer of BiIA-SG using an AAV vector was able to eliminate infected spleenocytes in 42% (5/12) of the HIV-1-infected humanized mice treated with AAV-BiIA-SG in middle or high doses in the absence of cART or...
latency-reversing agents. These findings are promising because in combination with cART, a higher dose of $2.5 \times 10^{10}$ gc AAV$_{10-1074}$ and AAV$_{BHCN117}$ was required to prevent 6 of 7 and 3 of 5 humanized mice from viral rebound, respectively (22). Moreover, a dose of $1 \times 10^{10}$ gc AAV$_{VRCO1}$ was necessary to provide protection in 7 of 10 humanized mice against HIV-1 JR-CRF challenge (17). Our data, therefore, demonstrated a proof-of-concept that in the absence of cART or latency inducers, AAV-BiIA-SG monotherapy at a dose of $4 \times 10^{10}$ gc or higher results in HIV-1 control and potentially effectively eliminates infected splenocytes in humanized mice.

Besides the cross-linking interactions with HIV-1 gp120 as mentioned above, we cannot exclude other mechanisms that may also contribute to the enhanced anti–HIV activity of BiIA-SG. In regard to neutralizing viruses that are resistant to both PGT128 and Hu5A8, one possible mechanism is that BiIA-SG may confer simultaneous blockade of HIV-1 gp120 and host receptor CD4. Because BiIA-SG has an enhanced anti–HIV potency compared with PGT128 and Hu5A8 in combination, BiIA-SG may also allow the enrichment of the PGT128 domains at the portal of viral entry for more efficient inhibition, as suggested for other bs-bnAbs (28, 30). In addition, it is possible that the binding of BiIA-SG Hu5A8 domains to CD4 increases the local concentration of PGT128 near the cell membrane for enhanced HIV-1 neutralization. This mechanism is possible because antibody membrane anchoring can increase neutralizing activities of both bnAbs and nonneutralizing anti–HIV antibodies (55, 56). Future structural analysis of BiIA-SG is necessary to answer its mode of action. Comparing the therapeutic efficacy of the cART+BiIA-SG regimen with the AAV–BiIA-SG regimen, we speculate that AAV–BiIA-SG provides a relatively prolonged and increased in vivo supply of functional BiIA-SG, and therefore, is more effective at the elimination of infected splenocytes in humanized mice.

It should be noted that HIV-1–infected humanized NSG-HuPBL mice do not fully represent infected patients, although this model has been widely used to study bnAb-based HIV-1 immunotherapy (4, 16, 18). To this end, there are no measurable host immune responses against BiIA-SG and the inoculated viruses. Moreover, besides infected T cells, HIV-1 latency in other cell types remains to be evaluated (41). These caveats need to be carefully investigated in the future using relevant models. For example, the humanized bone marrow–liver–thymus (BLT) model could be reconstituted with a broader range of human T cells, B cells, monocytes, and macrophages (57, 58). Human T cell development in transplanted thymus could be observed in the BLT model. Moreover, the humanized myeloid-only mice would be useful to study HIV-1 persistence in tissue-resident macrophages during bnAb immunotherapy (59). Since humanized mice have some limitations, such as incomplete human immune cells, lack of human lymph nodes, and difficulty in generating human antibody and memory T cell immune responses, future BiIA-SG efficacy studies are merited in immune competent nonhuman primate models such as simian human immunodeficiency virus–infected rhesus macaques or directly in human trials (24, 53).

**Methods**

**Engineering of IA and BiIA.** Genes encoding single chains (scFv) of bnAbs including PG9, PG16, PGT128, VRCO1, and Hu5A8 were prepared by automated codon optimization and DNA synthesis according to published sequences (7–9). Each scFv was constructed as a VL linked to a VH via a 15-mer (Gly$_5$Ser$_{10}$) linker (Figure 1). We fused scFvs of these bnAbs to the human immunoglobulin CH2-CH3 (hIgG1-Fc, containing an E333V mutation according to the EU numbering) region. To create secretory antibodies, the secretory signal peptide of tissue plasminogen activator (tpa) was linked to the N-termini of the IAs using PCR-based techniques to enhance the protein expression and release. Two genes encoded BiIA-DG, each with 2 corresponding residue mutations to promote the dimerization of electrostatically matched Fc chains using the knobs-into-holes method (25, 37). BiIA-SG was engineered by fusion of scFv-PGT128 to the 5’ end of the scFv-Hu5A8-hlgG1-Fc backbone with a 20-mer (Gly$_5$Ser$_{10}$) linker in between (Figure 2). Because we used the original sequence of Hu5A8 but not theibalizumab (28, 60), we kept the name Hu5A8 in this study.

**IA or BiIA expression and purification.** 293T cells (ATCC) were transfected with plasmids encoding various IA or BiIA genes and cultured for 72 hours at 37°C in a 5% CO$_2$ incubator after the transfection. IA-containing culture supernatants were harvested and centrifuged at 1,000 g for 10 minutes. IAs were purified immediately by affinity chromatography using Protein G–Agarose (Life Technologies) according to the manufacturer’s instructions. The purified IAs and BiIAs were concentrated by an Amicon ultracentrifuge filter device (molecular weight cutoff; 50 kDa; Millipore) to a volume of 0.2 ml in PBS (Life Technologies), and stored at −80°C. Only BiIA-DG was produced by cotransfection of 293T cells using equal amounts of 2 expression vectors.

**Western blot analysis.** The purity and molecular weights of IAs and BiIAs were analyzed by SDS-PAGE separating gels with 10% SDS-PAGE separating gels. Western blot analysis was performed on nitrocellulose membranes. The separated proteins were transferred onto PVDF membranes (Millipore) for antibody staining. The reducing and nonreducing conditions refer to the presence and absence of β-mercaptoethanol in the gel-loading buffer. The secondary antibody was an IRDye 800CW-conjugated goat anti-human IgG-Fc domain antibody (catalog 926-32232, Rockland). Protein bands were visualized using the Odyssey Image System (Li-COR).

**Binding specificity of IA and BiIA by ELISA.** Each well of high-binding 96-well plates (Costar) was coated with 50 ng gp120 or scCD4 overnight. After washing, the plates were blocked for 2 hours with the blocking buffer containing 2% BSA (catalog 10857, Affymetrix), 1 μg/ml EDTA (catalog AM9260G, Life Technologies), and 0.05% Tween-PBS (catalog Sc-29113, Santa Cruz) and then incubated with serially diluted IA or BiIA for 60 minutes at 37°C. After washing, the HRP-conjugated goat anti-human detection antibody (catalog sc2907, Santa Cruz Biotechnology) was added for 1 hour as previously described (61), followed by washing and addition of 100 μl HRP chromogenic substrate 3,3’,5,5’-Tetramethylbenzidine (TMB) (catalog 86036, MilliporeSigma). The ODs were measured at 450 nm using the VICTOR3 1420 Multilabel Counter (PerkinElmer). The background values given by incubation of PBS alone in coated wells were subtracted. The positivity was determined when the OD value was 2-fold above the negative controls (e.g., normal serum). All experiments were performed in duplicate.

**Surface plasmon resonance (SPR) analysis.** The binding kinetics/affinity of IA and BiIA to gp120 and scCD4 were tested in PBS running buffer at 25°C by SPR analysis using a Biacore X100 machine (GE Healthcare) as previously described (25). HIV-1$_{JR-CRF}$ gp120 and scCD4 proteins (30 μg/ml) were covalently coupled to CM5 sensor chips (GE Healthcare) by amine chemistry at pH 5.0, resulting in an immobiliza-
HIV-1 neutralization assay. The Global Panel HIV-1 Env clones were obtained from the NIH AIDS Reagent Program (catalog 12670) (40). The in-house panel of 40 Env clones was generated by us or provided by our collaborator (H Shang, China Medical University, Shenyang, China). A standard inoculum of 200 TCID$_{50}$ of each pseudovirus was used to test the potency and breadth of various neutralizing antibodies as previously described (62–64). Pseudovirus neutralization was measured using a luciferase-based assay in GHOST(3)-X4R5 (catalog 3942, NIH AIDS Reagent Program) (35, 38). Each IA and BiIA was tested in duplicate with a 3-fold serial dilution. The 50% and 90% inhibitory concentration (IC$_{50}$ and IC$_{90}$) of each IA and BiIA were calculated to reflect anti-HIV-1 potency. The synergistic effects of paired IAs were measured by a checkerboard synergy assay and calculated using the MacSynergy II software as previously described (36). Accordingly, the synergy volumes of –50 to 50, 50 to 100, and >100% in a synergy plot at the 95% CI were used to define additive, slight synergistic, and highly synergistic effects, respectively. For live HIV-1 neutralization, a standard viral inoculum of 200 TCID$_{50}$ was used for the assay in TZM-bcl cells (catalog 8129, NIH AIDS Reagent Program).

**Pulse BiIA-SG quantification by ELISA.** For ELISA, 25 ng HIV-1-p24, gp120 in coating buffer (pH 9.6) was used to coat 96-well plates (Costar) at 4°C overnight. After washing, the plates were blocked for 1 hour with blocking buffer (5% skim milk in PBS) and then incubated with serially diluted BiIAs or BiIA-containing sera for 60 minutes at 37°C. After washing, goat anti-human secondary antibody conjugated with HRP (catalog sc2907, Santa Cruz Biotechnology) was added for 1 hour, followed by washing and adding 100 μl HRP chromogenic substrate 3,3′,5,5′-TMB (catalog T4444, MilliporeSigma). ODs were measured at 450 nm using the VICTOR3 1420 Multilabel Counter (PerkinElmer). All experiments were performed in duplicate.

**Humanized mouse model.** Animal procedures that might cause more than slight pain or distress were performed with appropriate sedation or anesthesia. Immunodeficient NSG mice were purchased from the Jackson Laboratory (catalog 005557). Humanized NSG-HuPBL mice were generated from 4- to 6-week-old NSG mice as we previously described (41).

**Purity and half-life of BiIA-SG.** The purity of BiIA-SG was measured by size exclusion-high-performance liquid chromatography (SEC-HPLC). BiIA-SG purified from CHO cells was injected i.p. into 4 NSG-HuPBL mice at a dose of 20 mg/kg. ELISA was used to measure the serum concentration of BiIA-SG over a period of 7 days. The t$_{1/2}$ of BiIA-SG was computed as ln (2)/k, where k is a rate constant expressed reciprocally of the x axis time units by the 1-phase decay equation in the GraphPad software.

**Intestinal flush and homogenates preparation.** A single dose of 10 mg/kg BiIA-SG was i.p. injected into 4 NSG-HuPBL mice. Mucus, intestinal homogenates, and plasma specimens were collected 24 hours later as previously described (65). Sections (4 cm) from the small and large intestines were excised from NSG-HuPBL mice and flushed with 5 ml PBS using an 18-gauge needle (catalog NN-1825R, Terumo). Any remaining mucus was then manually extruded into the collection tube. The raw flush was vortexed for 15 seconds and centrifuged for 5 minutes at 16,000 g and the supernatant was separated. The flushed intestines were weighed and homogenized with 100 μl PBS. The homogenate was centrifuged for 5 minutes at 16,000 g and the supernatant was separated. The antibody concentration in small intestine and large intestine mucus and homogenate was detected by ELISA.

**Fluorescence-activated cell sorting (FACS) analysis.** Blood samples were collected from the facial veins of mice in Eppendorf tubes containing 50 μl anticoagulant (0.5 M EDTA) and were then centrifuged for 5 minutes at 1,150 g in a microcentrifuge. The plasma was stored for future analysis, and the cell pellets were resuspended in 2 ml of 1× RBC lysis buffer (BD Bioskience) and incubated on ice for at least 10 minutes to remove red blood cells. After the lysis, the cells were pelleted at 1,150 g in a microcentrifuge for 5 minutes at room temperature and stained for 60 minutes at 4°C with 100 μl of a cocktail containing 2 μl anti-human CD3-FITC (catalog sc37590, Santa Cruz Biotechnology), 1 μl anti-human CD4-PerCP-Cy5.5 (catalog sc47284, eBioscience), 1 μl anti-human CD8-PE (catalog sc4706, BD Bioscience), and 1 μl anti-human CD45 PE/Cy7 antibodies (catalog sc30416/H130) (all from BioLegend). The samples were washed with PBS supplemented with 2% fetal bovine serum and then were centrifuged at 800 g in a microcentrifuge for 5 minutes. The pelleted cells were resuspended in 300 μl wash buffer and analyzed on a FACSARia III flow cytometer (Becton-Dickinson). The samples were gated for human CD45 expression before analyzing the T cell subsets based on the CD3, CD4, and CD8 markers within this subset.

**Immunofluorescence (IFA) staining of HIV-infected cells in tissues.** Spleen and other tissues were immersed in 10% neutral buffered formalin (catalog Z2902, MilliporeSigma) for 24 hours. After the formalin fixation, the tissues were placed in 70% ethanol (Merck) and subsequently embedded with paraffin. Tissue sections (4-μm thick) were used for immunohistochemical staining for HIV-P24 detection using the Kal-1 murine monoclonal antibody (catalog M085701, Dako) as we previously described (41). Confocal images were obtained with a Carl Zeiss LSM 700 microscope using the ZEN 2012 software.

**Viral RNA load measurement by quantitative RT-PCR (qRT-PCR).** Viral RNA was extracted using the QiAamp viral RNA mini kit (Qiagen). Each RNA sample was reverse transcribed to 20 μl cDNA with the RT-PCR Prime Script Kit (Takara). The cDNA (2 μl) was used in a 20 μl qRT-PCR reaction with the TaqMan Universal PCR Master Mix (Life Technologies), a TaqMan probe (5′-FAM–CTCTCT CTTTCT AGCCTC–MGB-3′), and primers designed to target the P17 gene of HIV-1 (5′-TACTGA CGCTTCT CGACCC–3′ and 5′-TCTCGA CGCAGG ACTCG-3′). The samples were run in triplicate on an Eppendorf Realplex4 Mastercycler (Eppendorf). The following cycling conditions were used: 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes. The virus titer was determined by comparison with a standard curve generated using RNA extracted from a serially diluted reference viral stock. The limit of detection was 500 copies per milliliter for HIV-1$_{p24}$ plasmids and HIV-1$_{p24}$.  

**HIV-1 proviral DNA measurement by digital PCR.** Cell-associated HIV-1 proviral DNA was quantified by QuantStudio 3D digital PCR System (Life Technologies). Briefly, total cellular DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) and eluted in 40 μl volume. Purified DNA was quantified for HIV-1 proviral DNA by the QuantStudio 3D Digital PCR System using the primers, probe, and reaction condi-
tions as mentioned above for post-RT HIV-1 RNA quantification. Human CCR5 DNA was quantified with primers (5’-ATGAT TCCTG GGAGA GACGC-3’ and 5’-AGCCA GGAGC GTCCAC CTT-3’) and the sequence-specific probe (5’-VIC-AACAC AGCCA CCACG CAAGT CATCA-3’). All PCR reactions contained 7.25 µl Quantstudio 3D digital PCR master mix v2 in 14.5 µl reaction volume. HIV-1 proviral load is reported as copies per cell justified by cell numbers from CCR5 copies.

In vivo efficacy of BiIA-SG in humanized mice. For PrEP experiments, 1 day before HIV-1 challenge, blood samples from NSG-HuPBL mice were subjected to flow cytometry to determine the baseline CD4+/CD8 ratios. On the following day, 200 µg (10 mg/kg) BiIA-SG was injected i.p. into NSG-HuPBL mice (44). Four hours later the mice were challenged through the i.p. route with 10 ng P24 of live HIV-1 JJR-FL (466 TCID50) or HIV-1 BJZS7 (640 TCID50) diluted in 100 µl PBS (41). These mice were subjected to weekly blood sampling to monitor viral load, CD4/CD8 ratios, and P24+ cells, and finally were sacrificed for the detection of infected cells throughout the body. For cART treatment experiments, NSG-HuPBL mice were challenged through the i.p. route with 10 ng P24 HIV-1 JJR-FL. Four days after HIV-1 challenge, mice were subjected to 4 different treatments: cART+BiIA-SG, cART, BiIA-SG, and placebo. The cART cocktail was prepared as previously described (22). Individual tablets of tenofovir disoproxil fumarate (TDF, Gilead), Lamivudine (3TC, Shandong Weifang Pharmaceutical Factory Co.), and Raltegravir (RAL, MSD Pharmaceuticals) were triturated into fine powder and suspended in 100 µl PBS. The cART cocktail (TDF 2.46 mg, 3TC 1.48 mg, RAL 1.23 mg) was administered by daily oral gavage to each mouse. BiIA-SG was i.p. injected (400 µg per mouse) every 4 days. All mice were subjected to weekly blood sampling to monitor viral load and were sacrificed 63 days after HIV-1 challenge.

AAV–BiIA-SG production and quantification. AAV–BiIA-SG production was conducted as previously described (15). An AAV Helper-Free System was purchased from Cell Biolabs. The pAAV-MCS plasmid containing the BiIA-SG transgene or the pAAV-IREs-hrGFP control was cotransfected into AAV-293T cells together with the helper vector pHELP (catalog 240071, Agilent Technologies) and pAAV-DJ (catalog VPK-430-DJ, Cell Biolabs) at a ratio of 1:1:1 using the polyethylenimine (PEI) transfection reagent (Polysciences Inc.) in Opti-MEM medium (Gibco). The AAV–BiIA-SG supernatant was collected at 48, 72, 96, and 120 hours after transfection, concentrated with 5x polyethylene glycol (PEG) 8000 (catalog 89510, MilliporeSigma) and finally purified by 1.37 g/ml cesium chloride centrifugation. The final AAV–BiIA-SG supernatant was collected at 48, 72, 96, and 120 hours after transfection, concentrated with 5x polyethylene glycol (PEG) 8000 (catalog 89510, MilliporeSigma) and finally purified by 1.37 g/ml cesium chloride centrifugation. The final AAV–BiIA-SG stock was dissolved in PBS, aliquoted, and stored at -80°C. Purified AAV–BiIA-SG was quantified by qPCR as previously described (15). Briefly, a frozen aliquot of AAV–BiIA-SG was treated with DNase I (MilliporeSigma) for 30 minutes at 37°C, and then quantified with SYBR Premix Ex Taq II (Takara) and primers specific for the CMV enhancer (AAV-Mono-CMV-F: CCATTG ACGTCA ATGGGT GAGT and AAV-Mono-CMV-R: GCCAAG TAGGAA AGTCCC ATAAGG) or the BiIA-SG transgene (AAV-mono-F 383-406: GGACTC AGCTGCTG GTTGGT CACTGTCA GGCGGC and AAV-mono-R 486-406: ACCCTT TCGGCC AGTGAG AC). The samples were tested in triplicates on the ViiA 7 instrument for the AAV–BiIA-SG transgene (AAV-mono-F 383–406: GGACTC TGGTCA CTGTCA and AAV-mono-R 486–406: ACCCTT TCGGCC AGTGAG AC). The samples were tested in triplicates on the ViiA 7 instrument.

Statistical analysis. All qRT-PCR data were analyzed with GraphPad Prism 5.01 software (GraphPad Software). Two-tailed Student’s t tests were used for group comparisons. P < 0.05 was considered statistically significant with necessary mean ± SEM or mean ± SD.

Study approval. All animal experimental procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 2786-12) at the Laboratory Animal Unit of The University of Hong Kong. The Laboratory Animal Unit is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Author contributions. JG and ZC designed and invented BiIA-SG. XW, JG, MN, and MA performed most experiments and analyzed the data. KSL, YD, JL, TW, LC, HYT, LL, QZ, Hua Wang, and QW provided technical assistance and did animal experiments. Hui Wang, HS, LZ, and PZ provided specimens and viruses. XJ coordinated the bioreactor production. ZC designed and conceived the study, analyzed the data, and wrote the manuscript.

Acknowledgments. We thank the NIH Reagent Program for providing reference bnAbs and the global panel of Env clones. We thank National Natural Science Foundation of China (NSFC) and Hong Kong Research Grants Council (RGC) for the joint grant N_HKU709/11 (to PZ and ZC); Health and Medical Research Fund (HMRF12110952), RGC (HKUS/CREF/15G), Innovation and Technology Fund (ITS/170/17) and the University of Hong Kong Seed Fund (201611160018) (to ZC); China’s National Science and Technology Major Project (2013ZX10001005002001) (to ZC and XJ); NSFC grant 81530065 (to LZ); The Grand Challenge China (81661128042) (to LZ and ZC); the University of Hong Kong Development Fund and The Li Ka Shing Faculty of Medicine Matching Fund (to AIDS Institute); and the San-Ming Project of Medicine in Shenzhen (to ZC and Hui Wang).

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