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# Broadly Neutralizing Human Immunodeficiency Virus Type 1 Antibody Gene Transfer Protects Nonhuman Primates from Mucosal Simian-Human Immunodeficiency Virus Infection

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## ABSTRACT

**Broadly neutralizing antibodies (bnAbs) can prevent lentiviral infection in nonhuman primates and may slow the spread of human immunodeficiency virus type 1 (HIV-1). Although protection by passive transfer of human bnAbs has been demonstrated in monkeys, durable expression is essential for its broader use in humans. Gene-based expression of bnAbs provides a potential solution to this problem, although immune responses to the viral vector or to the antibody may limit its durability and efficacy. Here, we delivered an adeno-associated viral vector encoding a simianized form of a CD4bs bnAb, VRC07, and evaluated its immunogenicity and protective efficacy. The expressed antibody circulated in macaques for 16 weeks at levels up to 66 μg/ml, although immune suppression with cyclosporine (CsA) was needed to sustain expression. Gene-delivered simian VRC07 protected against simian-human immunodeficiency virus (SHIV) infection in monkeys 5.5 weeks after treatment. Gene transfer of an anti-HIV antibody can therefore protect against infection by viruses that cause AIDS in primates when the host immune responses are controlled.**

## IMPORTANCE

**Sustained interventions that can prevent HIV-1 infection are needed to halt the spread of the HIV-1 pandemic. The protective capacity of anti-HIV antibody gene therapy has been established in mouse models of HIV-1 infection but has not been established for primates. We show here a proof-of-concept that gene transfer of anti-HIV antibody genes can protect against infection by viruses that cause AIDS in primates when host immune responses are controlled.**

Broadly neutralizing antibodies (bnAbs) arise in human immunodeficiency virus type 1 (HIV-1)-infected individuals to various degrees (1–3), but vaccination to elicit such antibodies remains a challenge (4–6). An increasing number of potent bnAbs have been isolated in recent years from HIV-infected individuals (7–12). These bnAbs represent potential components for passive immunization in humans based on the finding that they protect nonhuman primates at physiologically achievable concentrations (13–16).

The transduction of long-lived cells with a viral vector encoding the heavy and light chain genes of bnAbs, also known as vectored immunoprophylaxis, aims to protect against HIV-1 infection by conferring expression of protective antibodies (17–20). In particular, viral vectors derived from adeno-associated virus (AAV) have yielded sustained expression of multiple bnAbs in mice (17, 20). These bnAbs confer neutralizing activity in the plasma of the mice and thereby protect humanized mice against intraperitoneal, intravenous, and mucosal HIV-1 challenge (20, 21).

Nonhuman primate models of HIV-1 infection represent the most appropriate model to assess the ability of antibodies to protect against infection (22–24). The comparable physiology of mucosal tissues, their relatively large size, and their similar immune system (25) give nonhuman primates distinct advantages over humanized mice for assessing the potential to protect against mucosal transmission of HIV-1 in humans. However, in the context of gene delivery, antibody persistence, localization, and protection against mucosal infection have not been well studied in nonhuman primates. A previous study conducted with nonhuman primates advanced this concept by using recombinant AAV1 vectors to deliver genes encoding antibody-like molecules called immunoadhesins (26). The immunoadhesins were expressed for >1

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year after gene transfer and protected most macaques against intravenous simian immunodeficiency virus (SIV) challenge (26, 27). It has yet to be shown whether full-length human antibodies composed of natural heavy and light chains can be delivered through vectored gene transfer to similarly prevent simian-human immunodeficiency virus (SHIV) infection in nonhuman primates. Additionally, despite being derived from a macaque antibody sequence, the recombinant immunoadhesins were immunogenic in 33% of the macaques expressing them (26). Later studies showed that immunoadhesins possess reduced neutralization activity compared to that of full-length antibodies (28). Thus, expression of full-length antibodies in primates remains a goal for clinical development of vectored immunoprophylaxis.

Long-term functional analysis of human bnAbs in nonhuman primates can be complicated by macaque immune responses against the human antibody (29, 30). In our previous studies examining the durability of protection by passive transfer of antibody protein, we observed macaque responses within weeks against the human antibody in >50% of macaques administered a single dose of 20 mg/kg of body weight of human VRC01 IgG (31). The immunogenicity of human proteins in nonhuman primates is a limitation of this model system, but it is not predictive of or relevant to immunogenicity in humans (30). The immunogenicity of human VRC01 was reduced by simianizing the antibody to create a macaque version of VRC01, which, when administered at 20 mg/kg, produced no detectable anti-VRC01 response (31). Although AAV8-vectored gene transfer has been proposed to reduce the immunogenicity of the transgene (32), human proteins such as factor IX elicit a humoral response in macaques when delivered by AAV8 vectors (33). The use of two immunosuppressants, cyclosporine (CsA) and rituximab, suppressed the macaque humoral response targeting factor IX, allowing the assessment of its function in macaques (33). We reasoned that it might be possible to control the immunogenicity of human antibodies in nonhuman primates using the above-mentioned approaches, allowing the examination of protection by AAV8-vectored bnAb gene transfer.

In this study, we assess the ability of recombinant AAV8 vectors to deliver a neutralizing antibody to protect against mucosal SHIV-BaLP4 challenge in nonhuman primates. We administered AAV8 vectors encoding a simianized version of the bnAb VRC07 with and without transient immunosuppressive treatment in rhesus macaques and demonstrate the efficacy of vectored bnAb immunoprophylaxis to protect against mucosal SHIV challenge.

## MATERIALS AND METHODS

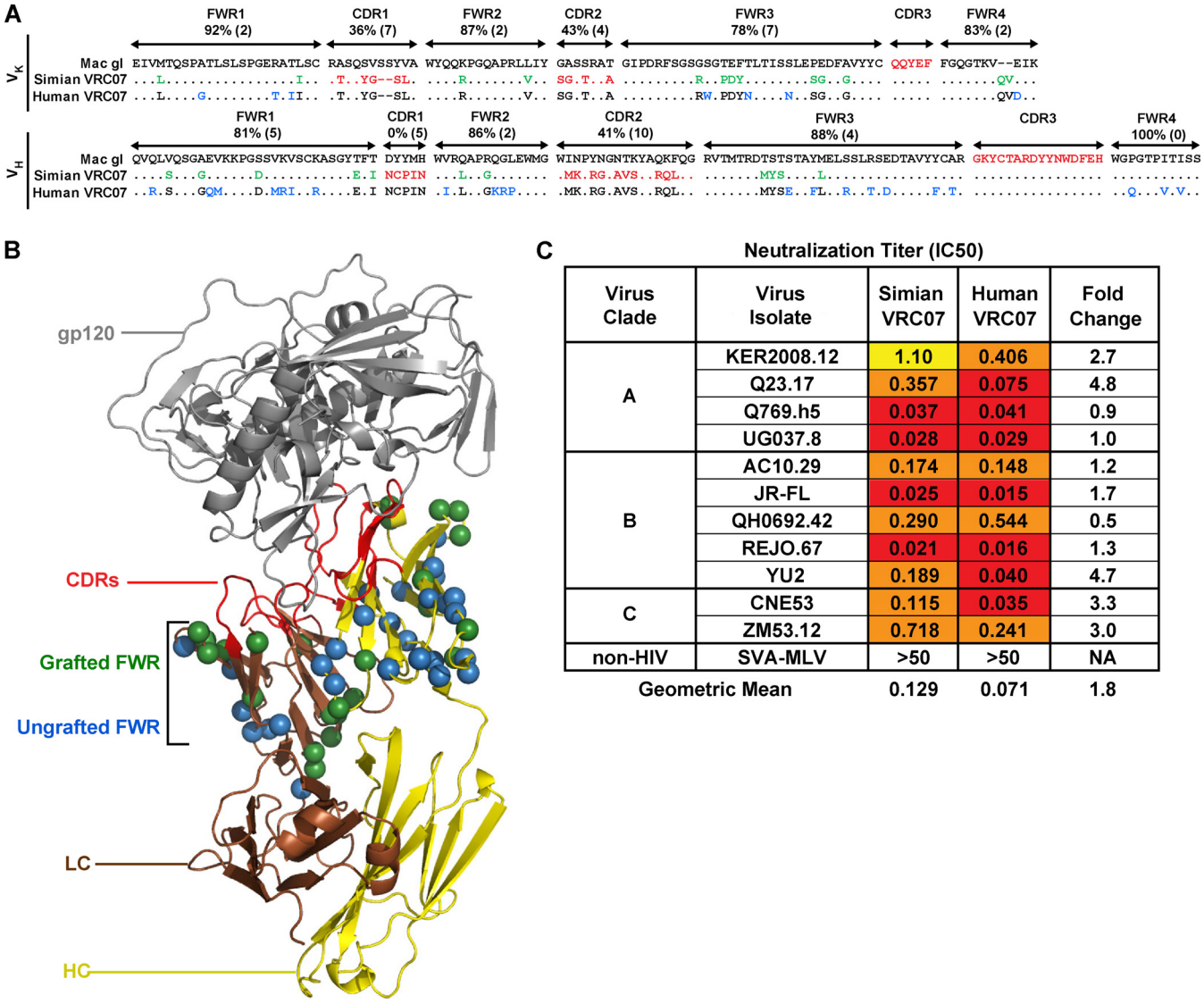
**Animals.** Indian-origin rhesus macaques (*Macaca mulatta*) weighing between 4 and 10 kg were used for nonhuman primate studies. The macaques were screened for AAV8 neutralizing antibodies prior to inclusion in AAV studies. Only macaques with plasma neutralization 50% inhibitory dilution ($ID_{50}$) titers of <1:20 were included. Macaques enrolled in SHIV-BaLP4 challenge studies weighed between 4 and 5 kg and were subjected to routine clinical evaluation. C.B-17 SCID mice were purchased from Taconic. Veterinary procedures were approved by the Vaccine Research Center Animal Care and Use Committee. All animals were housed and cared for in accordance with local, state, federal, and institute policies in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited facility.

**Simianization of VRC07 IgG.** Simianization of VRC07 was performed as previously described (31). This process transferred the entire complementarity-determining regions (CDRs) of human VRC07 (huVRC07) onto homologous macaque germ line genes that were chosen based on nucleotide sequence identity (Fig. 1A). The inferred macaque homologs were the same as those previously reported for VRC01, since VRC01 and VRC07 are derived from the same precursor B cell receptor (14, 31, 34). The inferred macaque variable gene homologs for the heavy and light chains were both 93% identical to the human germ line genes comprising VRC07. The selected macaque heavy and light chain J genes were 89 and 95% identical to the human germ line, respectively. Thirteen of the 20 and 11 of the 32 framework region (FWR) somatic mutations from the light chain and heavy chain of VRC07, respectively, were transplanted onto the homologous macaque germ line variable genes (Fig. 1A and B). These mutations were predicted to be important based on the crystal structure of VRC07 in complex with gp120 (14, 35) or have been implicated in correct CDR loop folding (36). In total, the FWRs of the light and heavy chain variable genes were 78 to 92% and 81 to 100% similar to macaque germ line gene sequence, respectively (Fig. 1A). To generate a full-length simianized immunoglobulin chain, macaque constant regions were appended after the variable region of the heavy and light chains.

**Antibodies.** Antibodies were made by transient transfection of 293F cells using 293fectin transfection reagent (Invitrogen). Six days after transfection, cell culture medium was clarified by centrifugation and filtration through a 0.2-μm filter. Antibodies were purified from the cell culture supernatant by using a protein A column on an Äkta fast protein liquid chromatography (FPLC) instrument (GE Healthcare). All purification equipment was pretreated with 1 N NaOH prior to use to eliminate endotoxin contamination. The purified antibody was dialyzed into phosphate-buffered saline (PBS) and tested for endotoxin using the *Limulus* amoebocyte lysate (LAL) assay (Lonza). Endotoxin above 1 endotoxin unit (EU) per mg of antibody was removed by EndoTrap column chromatography (Hyglos).

**Quantitative RSC3 ELISA.** Enzyme-linked immunosorbent assays (ELISAs) were performed by coating Nunc Maxisorp plates (Thermo Fisher) with 2 μg/ml of resurfaced HIV core derivative 3 (RSC3) (9) overnight at 4°C. The ELISA plates were blocked with a solution containing 5% skim milk and 2% bovine serum albumin (BSA) in Tris-buffered saline–0.05% Tween. To quantify a particular antibody in the plasma, a standard curve was derived by serially diluting purified simian VRC07 (simVRC07) or huVRC07 IgG in blocking buffer and adding it to the ELISA plate. Multiple dilutions of heat-inactivated macaque plasma were incubated in the plate for 1 h. Preimmune plasma was used to determine a baseline value for each macaque and was generally negative. Preimmune plasma spiked with a known concentration of huVRC07 or simVRC07 served as the positive control. Binding of simVRC07 or huVRC07 was detected with a horseradish peroxidase (HRP)-linked anti-macaque or anti-human Fc gamma antibody, respectively (Southern Biotech). The concentration of the VRC07 antibody in the macaque plasma was calculated based on a standard curve using Softmax Pro V5.0 software (Molecular Devices).

**Adeno-associated vector preparation.** The AAV8 vector was prepared as described previously, with the following modifications (20). Briefly, 293T cells were transfected with an AAV8 capsid plasmid, a transgene plasmid, and pHelp (Applied Viromics) at a 2:4:1 ratio by using the Profection kit (Promega). The cell growth medium was replaced with fresh Dulbecco's modified Eagle's medium (DMEM) (10% fetal bovine serum and 1% penicillin-streptomycin) 16 h after transfection. The cell culture supernatant was collected at 48, 96, and 120 h posttransfection, filtered with a 0.45-μm membrane, and stored at 4°C until purification of the vector. The vector was precipitated in 8% polyethylene glycol 8000 (PEG 8000) and 0.5 M NaCl for 2 to 16 h at 4°C. The precipitated vector was pelleted at 5,000 rpm for 30 min at 4°C and resuspended in 1.37 g/ml ultrapure cesium chloride (J. T. Baker). The vector was centrifuged for 24 h at 60,000 rpm in quick-seal tubes. After centrifugation, the tubes were punctured and fractionated into a 96-well plate. Fractions with a refractive index between 1.3730 and 1.3685 were found to contain approxi-




C

Neutralization Titer (IC50)

| Virus Clade | Virus Isolate | Simian VRC07 | Human VRC07 | Fold Change |
|---|---|---|---|---|
| A | KER2008.12 | 1.10 | 0.406 | 2.7 |
| | Q23.17 | 0.357 | 0.075 | 4.8 |
| | Q769.h5 | 0.037 | 0.041 | 0.9 |
| | UG037.8 | 0.028 | 0.029 | 1.0 |
| B | AC10.29 | 0.174 | 0.148 | 1.2 |
| | JR-FL | 0.025 | 0.015 | 1.7 |
| | QH0692.42 | 0.290 | 0.544 | 0.5 |
| | REJO.67 | 0.021 | 0.016 | 1.3 |
| | YU2 | 0.189 | 0.040 | 4.7 |
| C | CNE53 | 0.115 | 0.035 | 3.3 |
| | ZM53.12 | 0.718 | 0.241 | 3.0 |
| non-HIV | SVA-MLV | >50 | >50 | NA |
| Geometric Mean | | 0.129 | 0.071 | 1.8 |

**FIG 1** Design of simian VRC07 IgG. (A) Amino acid alignment of huVRC07 and simVRC07 heavy and light chain variable regions. Residues that are identical to the inferred germ line gene (Mac gl), shown on the top line, are indicated by periods. The complementarity-determining region (CDR) residues (red) and grafted framework region (FWR) residues (green) are shown. HuVRC07 CDR3 is shown below the germ line sequence, since the original sequences after recombination could not be predicted with a high degree of certainty. Somatic mutations in the FWRs that were not grafted to make simVRC07 are highlighted in blue. The percent similarity of each segment of simVRC07 compared to the macaque germ line is denoted above the sequences. The number of grafted residues is indicated in parentheses above each segment. (B) Tertiary sequence location of amino acids selected for engraftment onto the macaque germ line gene. Somatic mutations in the FWRs of the heavy chain (HC) and light chain (LC) of huVRC07 are shown as spheres on the crystal structure (14). The CDRs (red) and somatic mutations in the FWRs (blue or green) are colored to correspond to the alignment in panel A and are depicted in proximity to HIV-1 gp120 (gray). (C) Comparison of HIV-1 neutralization titers of simVRC07 and huVRC07 by a TZM-bl cell assay. Neutralization titers ($IC_{50}$s) (in micrograms per milliliter) of the indicated antibodies are shown and are color-coded as follows: red, <0.1; orange, 0.1 to 0.99; yellow, 1 to 4.9; green, 5 to 50; white >50. NA indicates that the fold change could not be calculated due to a lack of neutralization by both antibodies.

mately 90% of the vector. These fractions were pooled and subjected to a second round of cesium chloride density centrifugation using the procedure defined above. After a second round of centrifugation, the vector was fractionated and pooled again according to the same refractive index range. The vector was buffer exchanged into test formulation buffer 2 (10 mM Tris [pH 8], 100 mM sodium citrate) and stored at −80°C.

The vector titer was determined by absolute-quantification real-time PCR using Power SYBR green PCR mix (Invitrogen) and primers pAAVioUBCenhancer_F (GCCTTAGAACCCCAGTATCAG) and pAAVioUBCenhancer_R (ACTTTTCCTCGCCTGTTCCCG). The standard curve was generated by using the transgene plasmid that was used for vector production. The vector was treated with Turbo DNA-free DNase (Ambion) for 30 min at 37°C prior to inclusion in real-time PCR assay mixtures to eliminate DNA contamination from the transfection. Finally, vector purity was confirmed by SDS-PAGE and InstaBlue staining. All vectors prepared for *in vivo* administration were examined for endotoxin contamination using the EndoLISA assay (Hyglos) and were found to be below 1 EU per mg of vector protein.

***In vitro* AAV8 neutralization assays.** Twenty thousand 293T cells were plated into a 96-well plate 24 h prior to performing the assay. Macaque plasma was diluted 5-fold, starting at a 1:20 dilution. A total of $1 \times 10^{10}$ vector genomes (vg) of AAV8 encoding luciferase were incubated

with plasma or medium for 30 min at 37°C. The plasma and vector were then added to the plate containing 293T cells. Forty-eight hours later, luciferase was quantified by using the luciferase assay system (Promega) and a Victor3 luminometer (PerkinElmer). Percent neutralization was determined by the following equation: [1 − (luciferase expression in the cultures with plasma/luciferase expression in the cultures without plasma)] × 100. Percent neutralization curves were plotted in Prism version 6 (GraphPad), and $ID_{50}$s were calculated by fitting to a 4-parameter curve.

**Murine AAV gene transfer studies.** The AAV8 vector was diluted up to 100 μl with PBS. Four- or five-week-old C.B-17 SCID mice (Taconic) were anesthetized and injected with 50 μl of AAV8 vector in the gastrocnemius muscle of each leg. Serum was collected from the tail vein of mice and analyzed for VRC07 expression by quantitative RSC3 ELISA.

**Anti-F2A intracellular cytokine staining.** Intracellular cytokine staining of cryopreserved macaque peripheral blood mononuclear cells (PBMCs) was performed as previously described (37). Briefly, PBMCs were thawed and rested overnight. The following day, the PBMCs were stimulated for 6 h with 2 μg/ml of an overlapping pool of furin-2A (F2A) peptides in the presence of 10 μg/ml of brefeldin A. PBMCs that underwent the same process but that did not receive the peptide pool served as negative controls, and their values were subtracted from those of the corresponding treatment wells. The PBMCs were then stained with a viability dye and fluorochrome-conjugated antibodies against CD4, CD3, CD8, CD69, interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) for analysis on an LSRII flow cytometer (BD Biosciences). Positivity thresholds for each cytokine were previously determined by analysis of 100 seronegative samples.

**Anti-F2A plasma antibody ELISA.** ELISA plates were coated with 2 μg/ml of a pool of synthetic F2A peptides overnight at 4°C. The ELISA plates were blocked with a solution containing 5% skim milk and 2% bovine serum albumin in Tris-buffered saline–0.05% Tween. Heat-inactivated macaque plasma was incubated in the plate for 1 h at several dilutions. Binding of macaque IgG was detected with an HRP-linked anti-macaque Fc gamma antibody (Southern Biotech) and a tetramethylbenzidine (TMB) substrate (KPL). The absorbance at 450 nm in each well was determined with a Spectramax instrument (Molecular Devices) and Softmax Pro V5.0 software (Molecular Devices).

**AAV8 vector administration to nonhuman primates.** On the day of administration, AAV8 vectors were thawed on ice and diluted up to 1 ml in PBS. Macaques were anesthetized, and a preimmune blood sample was taken from the femoral vein. Subsequently, the macaque was administered a single injection of 1 ml of the AAV8 vector in the quadriceps muscle.

**Immune suppression in nonhuman primates.** At the initiation of the study (day −9), rhesus macaques were placed under general anesthesia and administered an injection of 5 mg/kg of cyclosporine (Sandimmune; Novartis). Cyclosporine was diluted in sterile PBS and infused intravenously at a rate of 1 ml/min for 60 min. This procedure was repeated the following day. Beginning on day −6 and ending on day 28, macaques were administered 15 to 30 mg/kg of a cyclosporine oral solution by oral gavage every other day. AAV8 encoding simVRC07 IgG was administered on day 0, as stated above. For quantification of cyclosporine blood concentrations, blood was obtained before drug administration and 1.5 to 2 h after drug administration. Cyclosporine was quantified in whole blood by using the Dimension Systems cyclosporine assay (Siemens Diagnostics), and its level was found to be >1,100 ng/ml post-drug administration.

**Mucosal SHIV challenge studies.** Macaques were sedated using general anesthesia, and a prechallenge blood sample was taken from the femoral vein. The hindquarters of the macaque were elevated, and 1 ml of SHIV-BaLP4 (12,800 50% tissue culture infective doses [$TCID_{50}$]/ml when the titer was determined in TZM-bl cells) (obtained from Norman Letvin) was delivered into the rectum (14, 15, 31, 38, 39). The hindquarters of each macaque were kept elevated for 15 min to promote absorption of the inoculum. To detect infection, the plasma viral load was determined weekly by quantitative PCR. Specifically, virions were lysed and RNA was isolated by using a QIAsymphony virus/pathogen midi kit (Qiagen). RNA was reverse transcribed with Superscript III (Invitrogen). Viral cDNA was quantified by real-time PCR in a StepOne PCR machine (Applied Biosystems), using a SIV Gag-specific TaqMan primer set.

**Monoclonal antibody blocking assays.** ELISA plates were coated with RSC3 and blocked with ELISA blocking buffer as stated above (9). A total of 0.4 μg/ml of huVRC07 or simVRC07 was incubated alone or with a 1:20 dilution of either week 0 or week 9 plasma for 30 min at 37°C. Human VRC03 was used as a negative-control antibody and incubated in the same manner with plasma. The treated monoclonal antibodies (MAbs) were then incubated in the RSC3-coated ELISA plates for 30 min at room temperature. Binding of the monoclonal antibody to RSC3 was detected with HRP-labeled anti-IgG Fc (Southern Biotech) and developed with a TMB substrate according to the manufacturer's protocol (KPL). The absorbance at 450 nm was read, and data were analyzed with Softmax Pro V5.0 software (Molecular Devices).

**Anti-simVRC07 biolayer interferometry binding assays.** A fortéBio Octet Red384 instrument was used to measure binding of plasma to control macaque IgG (NIH Nonhuman Primate Reagent Resource) and simVRC07 IgG. Each antibody (10 μg/ml) was immobilized by amine coupling to biosensors via activation in an ethyl-dimethyl-aminopropyl-carbodiimide–*N*-hydroxy-succinimide activation mixture for 300 s in 10 mM acetate (pH 5). The biosensor reactivity was quenched by using 10 mM ethanolamine (pH 8.5). Typical capture levels were between 0.7 and 1 nm, and the variability within a row of eight tips did not exceed 0.1 nm. Biosensor tips were then equilibrated for 300 s in 1% BSA–PBS prior to binding measurements. Plasma samples from macaques were diluted 1:50 in a 1% BSA–PBS solution, and binding to each immobilized antibody was assessed for 300 s. All the assays were performed with agitation set to 1,000 rpm in 1% BSA–PBS in order to minimize nonspecific interactions. The final volume of all solutions was 100 μl/well. Assays were performed at 30°C in solid-black 96-well plates (Greiner Bio-One).

**SimVRC07 reverse immunohistochemistry.** Pinch biopsy specimens were taken from the rectum of rhesus macaques before and 3 weeks after AAV8 injection. The biopsy specimens were fixed for 3 h at room temperature in SafeFix II (Fisher Scientific), washed three times with 80% ethanol, and then stored overnight in 80% ethanol. The fixed tissues were paraffin embedded until they were stained for simVRC07. Prior to staining for simVRC07, the tissue was deparaffinized, rehydrated, and then blocked with Sniper (Biocare Medical). To detect simVRC07 IgG, the tissue was incubated with fluorescein isothiocyanate (FITC)-labeled RSC3, blocked by hydrogen peroxide, and incubated with anti-FITC-HRP. Staining was developed with a diaminobenzidine peroxidase substrate kit (Biocare Medical), and the tissue was dehydrated and mounted onto slides.

***In vitro* HIV and SHIV neutralization assays.** Monoclonal antibody neutralization of HIV-1 isolates was assessed in single-cycle pseudovirus assays by using TZM-bl cells as target cells, as previously described (40, 41). Plasma neutralization of replication-competent SHIV-BaLP4 was performed similarly, using TZM-bl cells as targets. Briefly, macaque plasma was serially diluted in culture medium, and virus was added to a final volume of 50 μl. Virus and plasma were incubated for 30 min at 37°C, and target cells were then added to a final volume of 70 μl. After 48 h, luciferase expression by the TZM-bl cells was quantified for each well. Neutralization was quantified as the percent reduction in luciferase expression in the cultures containing plasma compared to luciferase expression in TZM-bl cells infected in the absence of plasma. Neutralization curves were fit by using a five-parameter nonlinear regression equation calculated with JMP software (SAS, Cary, NC). The neutralization titers were calculated as the concentration of monoclonal antibody or the plasma dilution that inhibited 50% of the virus replication measured in infected cells cultured alone ($IC_{50}$ or $ID_{50}$, respectively).

**Statistical analyses.** Statistical tests were performed by using GraphPad Prism version 6.0 (GraphPad, La Jolla, CA).

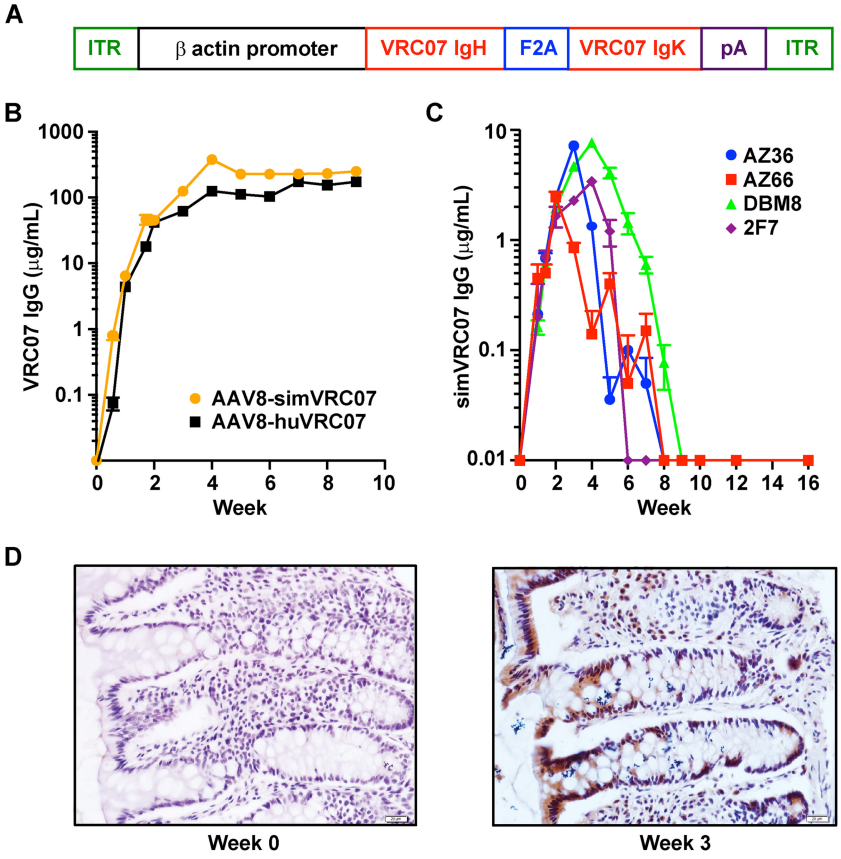



**FIG 2** AAV8 delivery of simVRC07 genes confers systemic and mucosal expression in immunodeficient mice and immunocompetent rhesus macaques. (A) Vector transgene design for single-stranded AAV8 vectors. ITR, inverted terminal repeat; pA, polyadenylation sequence. (B) Comparison of huVRC07 and simVRC07 serum concentrations in SCID mice after administration of $2.5 \times 10^{10}$ vg of AAV8 vectors. Values represent means ± standard errors of the means of results from three or six mice. (C) Plasma simVRC07 concentration in rhesus macaques administered $1 \times 10^{13}$ vg of AAV8-simVRC07. Values indicate means ± standard errors of the means of data from triplicate independent measurements. (D) Immunoperoxidase staining for simVRC07 IgG in cross sections of macaque rectal tissue by HIV envelope reverse immunohistochemistry. Rectal biopsy specimens were collected before and 3 weeks after AAV8-simVRC07 administration and stained with a labeled RSC3 protein. Original magnification, ×40. Images are representative of staining performed on tissues from macaques DBM8 and AZ36.

## RESULTS

**Simianization of VRC07 IgG.** The human bnAb VRC07 (huVRC07) was simianized to reduce its potential immunogenicity in macaques (14, 31) (Fig. 1A and B). We used a CDR grafting procedure that was previously shown to reduce the immunogenicity of human VRC01 in macaques (31). The simianization procedure yielded variable regions of each antibody chain that possess macaque framework regions with minimal transplanted huVRC07 residues and all three huVRC07 CDRs appended to macaque constant regions. To ensure that simianized VRC07 (simVRC07) retained the neutralizing capacity of huVRC07, we compared their activities in an *in vitro* TZM-bl pseudovirus assay. On a panel of 11 clade A, B, and C HIV-1 isolates, simVRC07 showed only a modest reduction in potency (1.8-fold), measured as the geometric mean $IC_{50}$ titer, compared to huVRC07 IgG (Fig. 1C). We were thus able to engineer a macaque version of huVRC07 that possessed neutralization activity similar to that of the wild-type human antibody.

**Systemic simVRC07 expression after AAV8-vectored gene transfer.** Gene delivery and expression of CD4-like molecules and immunoadhesins were previously demonstrated in macaques (26, 42). However, full-length antibodies have been expressed only by AAV-mediated gene transfer in mice (17, 18, 20, 43). To determine whether full-length antibodies could be expressed in nonhuman primates after intramuscular injection of a viral vector, we constructed single-stranded-DNA AAV8 vectors encoding either huVRC07 IgG (AAV8-huVRC07) or simVRC07 IgG (AAV8-simVRC07) (18–20). The transgene cassette utilized the furin-2A antibody expression system and removed all AAV open reading frames (Fig. 2A). The inverted terminal repeats of AAV2 flank each end of the transgene cassette to enable packaging of the heavy and light chain genes of VRC07 into viral vectors. The transduction activity of each vector was assessed by injecting $2.5 \times 10^{10}$ vector genomes (vg) of either vector intramuscularly into immunodeficient mice. After vector administration, the serum concentration of simVRC07 IgG peaked at 377 μg/ml 4 weeks after vector administration, which was higher than the concentration of huVRC07 IgG at the same time point. However, over the 9 weeks of follow-up, the serum concentrations of huVRC07 and simVRC07 were both maintained at ~200 μg/ml in the mice (Fig. 2B). These VRC07 concentrations were similar to the values previously reported for VRC01 (20).

To determine whether an AAV vector could support the expression of a transgenic full-length antibody in primates, $1 \times 10^{13}$

**TABLE 1** AAV8 plasma neutralization titers

| Animal | $ID_{50}$ | |
|---|---|---|
| | Wk 0 | Wk 3 |
| AZ36 | <20 | 3,994 |
| AZ66 | <20 | 176 |
| DBM8 | <20 | 4,269 |
| 2F7 | <20 | 303 |

vg of the AAV8-simVRC07 vector were administered to each of four rhesus macaques. After vector administration, we detected simVRC07 IgG in the plasma of all four macaques within 1 week (Fig. 2C). The plasma concentration of simVRC07 IgG peaked between weeks 2 and 4 in the macaques, at 2.5 to 7.7 μg/ml. However, simVRC07 IgG was undetectable in the plasma of all macaques by week 9. Similar expression kinetics were seen when human VRC07 or simian VRC01 was expressed instead of simVRC07 and when a 10-fold-lower dose of the AAV8-simVRC07 vector ($1 \times 10^{12}$ vg) was administered to macaques (data not shown). A single administration of the vector was given in each study because the macaques seroconverted for AAV8 neutralizing antibodies after vector injection (Table 1).

To determine whether simVRC07 IgG trafficked to gut mucosal surfaces when delivered by intramuscular AAV8 vector injection, we performed immunohistochemistry on rectal biopsy specimens using a labeled HIV Env core to detect simVRC07 IgG. Immunoperoxidase staining of week 3 rectal tissue revealed that simVRC07 IgG was capable of binding the envelope in epithelial cells in the crypts and bordering the rectal lumen (Fig. 2D). This staining was not seen in samples taken prior to treatment (week 0). The presence of simVRC07 IgG at this mucosal site indicated that the antibody produced from muscle cells trafficked to a mucosal site where it could potentially protect against virus transmission.

**Development of anti-idiotypic antibodies is associated with decreased simVRC07 expression.** To determine whether a macaque humoral response to simVRC07 was elicited by AAV8-simVRC07 administration, we assayed for binding antibodies against simVRC07 IgG. Specific antibodies to simVRC07 arose between 2 and 4 weeks after vector administration, and their levels increased over the 12 weeks of study (Fig. 3A). The magnitude of these anti-simVRC07 antibodies correlated inversely with the simVRC07 IgG plasma concentrations in the four macaques ($P < 0.0001$) (Fig. 3B). As expected, substantial antitransgene responses were also observed when AAV8-huVRC07 was administered to macaques (data not shown).

We asked whether these antibodies recognized the simVRC07 antigen binding site by analyzing whether the macaque plasma could block binding of simVRC07, huVRC07, or a control CD4

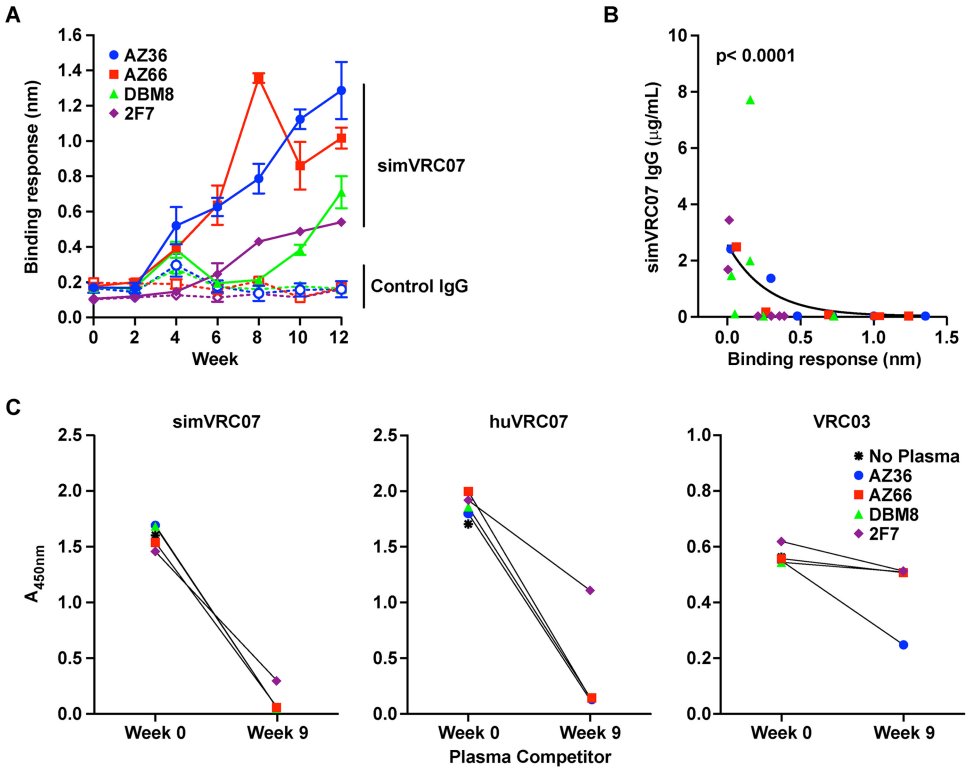



**FIG 3** Characterization of anti-simVRC07 humoral immunity in macaques. (A) Time course of anti-simVRC07 antibody levels measured by biolayer interferometry in macaques administered $1 \times 10^{13}$ vg of AAV8-simVRC07 (Fig. 2C). Plasma immunoglobulin binding to simVRC07 (solid lines) or control macaque IgG (dashed lines) is shown for each macaque. Values shown are means ± standard errors of the means from triplicate independent measurements. (B) Spearman correlation between the plasma simVRC07 concentration and the anti-simVRC07 plasma antibody response, quantified as described above for panel A (Spearman $r = -0.7819$). Symbols are coded to correspond to each rhesus macaque in panel A. Anti-simVRC07 responses were inversely correlated with detectable plasma simVRC07 concentrations. (C) Blocking of simVRC07, huVRC07, or VRC03 binding to HIV-1 gp120. Macaque plasma obtained before AAV8-simVRC07 vector administration (week 0) or 9 weeks after vector administration (week 9) was tested for blocking of the monoclonal antibodies. Binding in the presence of no plasma served as the baseline binding control. The means from triplicate measurements are shown.

binding-site antibody, VRC03, to a resurfaced stabilized core (RSC) of HIV-1 Env, RSC3 (9). Before vector administration, no blocking activity was detectable. In contrast, plasma from three of the four macaques blocked simVRC07 and huVRC07 binding to the HIV-1 envelope (Fig. 3C). No effect on a divergent CD4bs MAb, VRC03, was observed, suggesting an anti-idiotypic component. Plasma from one macaque inhibited binding by simVRC07, huVRC07, and, to a lesser extent, VRC03. The moderate blocking of VRC03 by this serum was possibly due to the recognition of framework residues shared between all three antibodies, since VRC07 and VRC03 are derived from the same $V_H$ gene. The binding of huVRC07 and its ability to block the interaction with HIV-1 Env indicated that the macaques generated antibodies to the variable antigen binding regions of simVRC07 and huVRC07. Therefore, in general, the administration of AAV8-simVRC07 vectors in macaques elicited antibodies targeting the huVRC07 residues in simVRC07.

**Plasma IgG and CD4$^+$ T cells against the furin-2A peptide arise after vector administration.** The F2A sequence used to connect the heavy and light chain genes contains the 2A sequence from foot-and-mouth disease virus and thus could potentially be immunogenic when expressed *in vivo*. Although we used an optimized version of the F2A sequence designed to eliminate this sequence from the antibody (19), it remained possible that T and B cells could react with the F2A peptide delivered in the AAV8 vector to macaques. For cellular responses, intracellular cytokines produced by CD4 and CD8 T cells after F2A peptide stimulation were assessed in cryopreserved PBMCs before AAV8-simVRC07 delivery and at two time points after vector delivery. None of the four animals possessed CD8$^+$ T cells that responded to F2A peptide stimulation (Fig. 4A). In contrast, macaque AZ36 possessed CD4$^+$ T cells after vector administration that produced IFN-γ, TNF-α, and IL-2 in response to F2A peptides (Fig. 4B). CD4$^+$ T cells isolated after AAV8 vector administration from macaque AZ66 were also stimulated by F2A peptides to produce TNF-α and IL-2 (Fig. 4B). These same two monkeys as well as an additional macaque possessed moderate levels of anti-F2A antibodies, which were not present 2 weeks after vector administration but were evident by week 8 (Fig. 4C).

**Suppression of host immunity increases simVRC07 expression following gene transfer.** Cross-species immunogenicity limits the persistence of human proteins in nonhuman primates (30). Given the high concentrations of simVRC07 expressed by the same AAV8 vectors in immunodeficient mice, we hypothesized that the vectors could function similarly in nonhuman primates in the absence of transgene immunity. We therefore attempted to suppress the macaque immune response to the transgene by administering cyclosporine (CsA). This approach was previously found to permit AAV-mediated expression of human factor IX in macaques (33). CsA was initially administered to six macaques 9 days prior to AAV8-simVRC07 vector administration. The plasma concentration of simVRC07 IgG increased in the macaques over the first 3 weeks, reaching a peak of 66 μg/ml in one macaque and a mean peak plasma concentration of 38.12 μg/ml for all six macaques (Fig. 5A and B). In one macaque, simVRC07 expression reached 41 μg/ml in plasma, highlighting an initial effect of the treatment, but antitransgene antibodies rendered simVRC07 undetectable despite ongoing CsA administration (Fig. 5A). SimVRC07 expression persisted in the remaining five macaques during the CsA regimen, and the peak concentrations of

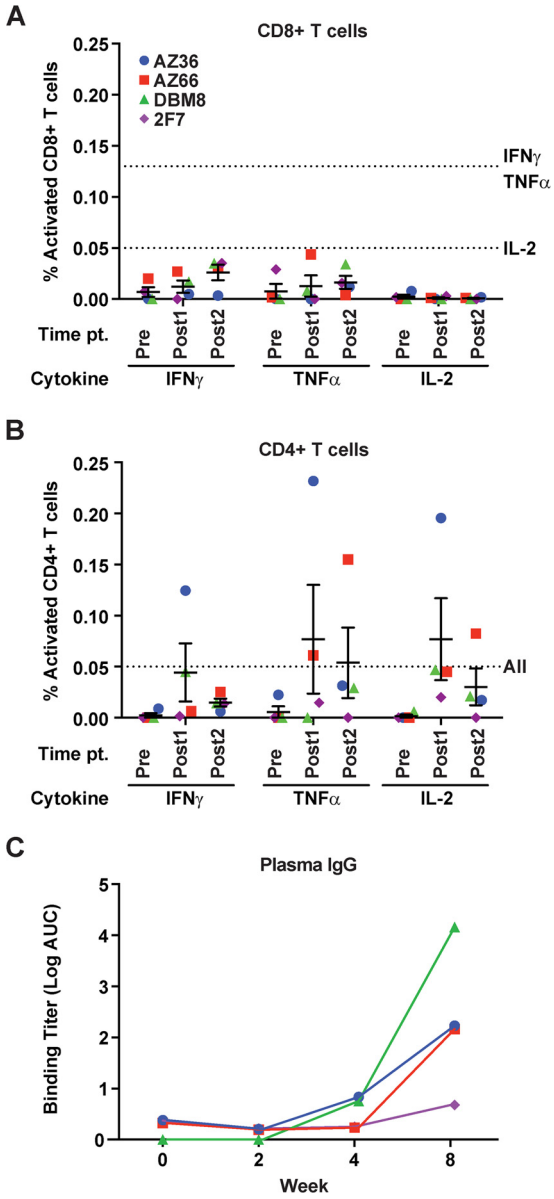



FIG 4 Cellular and humoral immune responses against the F2A peptide in macaques administered $1 \times 10^{13}$ vg of AAV8-simVRC07. (A and B) Intracellular cytokine staining of CD8$^+$ T cells (A) and CD4$^+$ T cells (B) stimulated with a pool of overlapping F2A peptides. The dotted line represents the previously determined threshold for positivity for each cytokine (37). Due to PBMC availability, T cell responses were analyzed at 12 and 25 weeks after vector administration for monkeys AZ36 and DBM8 and at 5 and 7 weeks after vector administration for monkeys AZ66 and 2F7. (C) ELISA of direct plasma IgG binding to pooled F2A peptides. The binding titer is represented as the $\log_{10}$ area under the curve (AUC) for the four macaques listed in panels A and B over time.

simVRC07 were significantly higher than those observed in macaques that were not immunosuppressed, although the macaques were not age and weight matched between groups ($P = 0.0095$ by a Mann-Whitney test) (Fig. 5B). Immune-suppressive treatment was halted 4 weeks after vector injection to determine whether simVRC07 expression would continue and to assess whether antitransgene responses would arise. The simVRC07 IgG plasma concentration decreased in all of the animals; however, three out

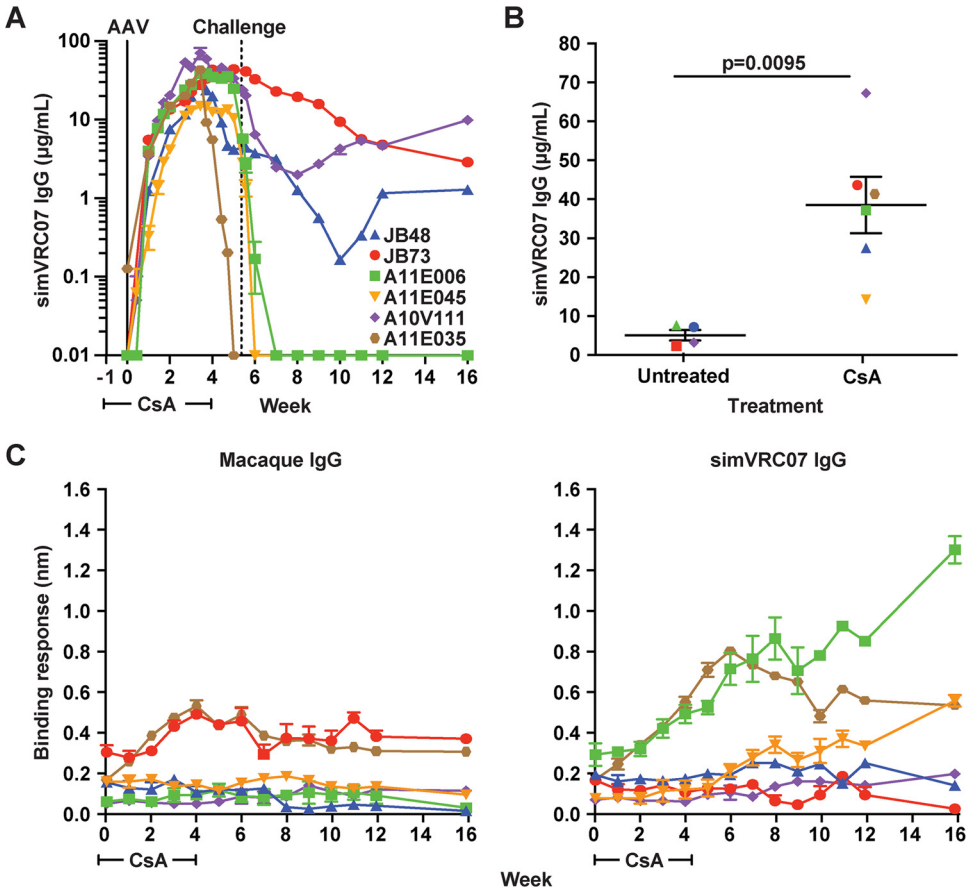



**FIG 5** Transient immune suppression during gene transfer increases peak simVRC07 concentrations and duration of expression. (A) SimVRC07 plasma concentrations in five rhesus macaques treated with cyclosporine (CsA), as indicated on the *x* axis. AAV8-simVRC07 was administered at week 0 (solid vertical line). The time point for SHIV-BaLP4 challenge is shown as a dashed vertical line. Values represent the means ± standard errors of data from triplicate independent measurements. (B) Comparison of peak plasma simVRC07 concentrations after AAV8-simVRC07 injection in untreated macaques (Fig. 2C) or the macaques treated with CsA listed in panel A. The mean peak concentration ± standard error of the mean for each group of animals is shown by the horizontal bars. Significantly higher peak plasma concentrations were observed for macaques that received CsA than for untreated macaques ($P = 0.0095$ by Mann-Whitney test). (C) Biolayer interferometry binding of plasma immunoglobulin from the macaques listed in panel A to macaque IgG (left) and simVRC07 IgG (right). The duration of CsA treatment is indicated on the *x* axis. Mean values ± standard errors of the means of data from triplicate independent measurements are shown.

of the six macaques maintained detectable simVRC07 IgG in the plasma for the following 16 weeks (Fig. 5A). Examination of transgene-specific antibodies showed that all three macaques in which simVRC07 was diminished to undetectable concentrations possessed antitransgene antibodies (Fig. 5C). In contrast, the three macaques with detectable circulating simVRC07 did not generate transgene-specific binding antibodies even after the cessation of immune-suppressive treatment (Fig. 5C).

**AAV8-simVRC07 confers protection against SHIV infection.** To determine whether AAV8 transfer of neutralizing antibody genes could protect macaques from mucosal SHIV infection, the monkeys given CsA and AAV8-simVRC07 were intrarectally challenged with CCR5-tropic SHIV-BaLP4 5.5 weeks after vector administration. The control group of macaques received a CsA dosing regimen identical to that of the treatment group and were administered an AAV8 vector encoding a simianized non-HIV antibody (AAV8-control IgG) on day 0. The plasma concentration of simVRC07 IgG on the day of challenge in the macaques given AAV8-simVRC07 ranged from 0 to 38.9 μg/ml (Fig. 5A). Similarly, for the macaques with detectable simVRC07, the *in vitro* neutralization titers ($ID_{50}$s) against the challenge stock varied from 1:31 to 1:1,028 (Fig. 6A). Upon challenge, all five macaques given AAV8 encoding control IgG became infected (Fig. 6B). Four out of six macaques administered AAV8-simVRC07 resisted infection ($P = 0.0455$ by Fisher's exact test) (Fig. 6B). Macaque A11E035 had no detectable simVRC07 in circulation and was expectedly one of the infected animals. The other infected macaque, A11E045, displayed the lowest peak and day-of-challenge concentrations of simVRC07 IgG among all of the macaques in the AAV8-simVRC07 group and also showed anti-simVRC07 plasma activity (Fig. 5A and C).

## DISCUSSION

Broad and potent neutralizing antibodies to HIV-1 provide a potential tool to prevent infection in the absence of an effective vaccine (13). Because passive infusion of antibodies requires multiple and sustained treatments, we explored whether HIV-1 bnAb gene delivery could protect nonhuman primates from mucosal SHIV infection. Transfer of full-length immunoglobulin genes in the absence of immune suppression yielded antibody concentrations

A

Plasma Neutralization Titer (ID50)

| Clade | Virus | JB48 | JB73 | A11E006 | A11E045 | A10V111 | A11E035 |
|---|---|---|---|---|---|---|---|
| B | SHIV-BaLP4 | 184 | 1028 | 31 | 34 | 257 | ND |
| non-HIV | SVA-MLV | <10 | <10 | <10 | <10 | <10 | ND |

B

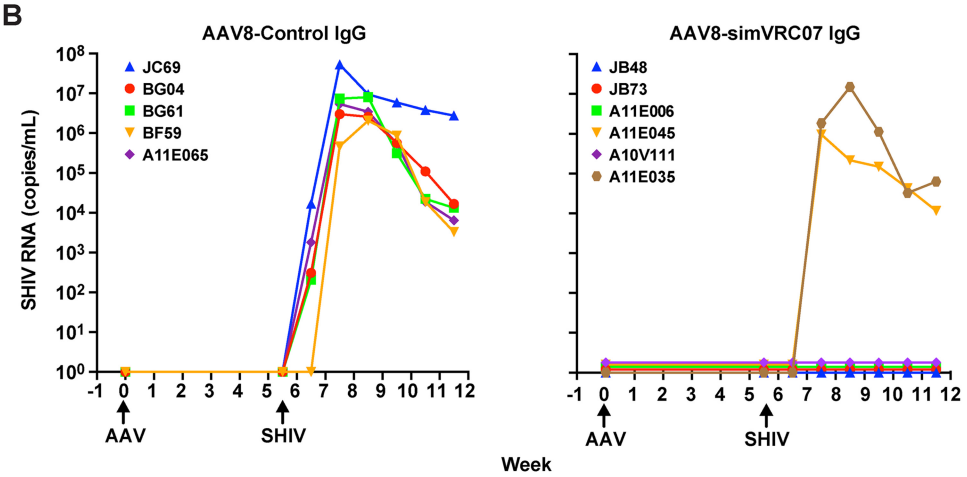



**FIG 6** SimVRC07 gene transfer protects against mucosal SHIV challenge. (A) Plasma neutralization titers ($ID_{50}$s) against the SHIV-BaLP4 challenge stock on the day of challenge. The neutralization titer in the plasma for monkey A11E035 was not determined (ND), since it did not have detectable simVRC07 in its plasma at this time point. The $ID_{50}$ is shown as the reciprocal plasma dilution and is color-coded as follows: red, >500; orange, 300 to 500; yellow, 100 to 299; green, 10 to 99; white, <10. Murine leukemia virus (SVA-MLV) was analyzed as a negative-control virus. (B) Plasma viral loads in rhesus macaques administered AAV8 vectors encoding control IgG (left) or simVRC07 (right) and challenged with SHIV-BaLP4 5.5 weeks later. Prior to SHIV-BaLP4 challenge, both groups of animals received the same course of CsA treatment beginning 1 week before AAV8 administration and ending at week 4. Significantly more macaques were infected in the control group than in the simVRC07 group (treatment group, $n = 6$; control group, $n = 5$ [$P = 0.0455$ by Fisher's exact test]).

of ~5 μg/ml on average in nonhuman primates, which was increased to an average of 38 μg/ml when macaques were immune suppressed at the time of vector delivery. In a previous study, Johnson et al. injected a single-stranded AAV1 vector encoding a soluble CD4 immunoadhesin and readily detected significant levels in macaques (26). In the present study, similar detectable levels were also reached with an AAV8 vector expressing full-length IgG, suggesting that AAV-vectored gene delivery in nonhuman primates is not limited to immunoadhesins. Johnson and colleagues observed concentrations approaching 200 μg/ml for two other immunoadhesins in macaques. These immunoadhesins differed from the CD4 immunoadhesin in that they were derived from natural macaque antibodies and were delivered with self-complementary AAV vectors (26, 44). The extent to which each of these differences accounts for the enhanced expression in macaques is presently unclear. Similar to the CD4 immunoadhesin, the present study used a single-stranded DNA AAV vector rather than a self-complementary AAV vector. The advantages of each vector format are still debated, particularly with regard to the size of the transgene that each vector carries and the expression level that each type affords.

The passive transfer of VRC01 protein suggests that the accumulation of antibody in mucosal tissue improves protection against mucosal SHIV challenge (38). Mucosal localization of antibodies produced by gene transfer presumably contributes to protective efficacy. Immunohistochemistry of the rectal tissue indicated that simVRC07 IgG expressed from intramuscular gene delivery localizes to mucosal tissue in a manner similar to that of a passively transferred bnAb protein (31, 38). Thus, it seems that once the transgenic antibody is expressed *in vivo*, it traffics to the mucosa, where it can counteract virus infection in a manner analogous to that of a passively transferred antibody protein.

Protection against mucosal infection in macaque models of HIV infection has been demonstrated after passive transfer of monoclonal antibodies (45–48), but it has not been shown previously after gene transfer of bnAbs. We show here that gene transfer of bnAb genes yields sufficient antibody expression in nonhuman primates to confer protection. Previous studies of passive protein transfer have shown that the probability of protection against viral challenge is related to the plasma antibody concentration (14, 49). In our previous studies with VRC01 and the same SHIV-BaLP4 challenge, we infused VRC01 at various doses and used a regression analysis to correlate the level of plasma antibody with *in vivo* protection (14, 15, 38). These data revealed that 50% protection was achieved at a plasma VRC01 concentration of 2.54 μg/ml. Monkeys A11E006 and A11E045 both had plasma concentrations of simian VRC07 in the 1- to 2-μg/ml range, and one of the two macaques became infected. This result is consistent with the probability of protection for VRC01-like antibodies being <50% at concentrations of <2.54 μg/ml (14, 15, 38).

The macaques in this study were treated with CsA prior to SHIV-BaLP4 challenge. Cyclosporine has been shown to inhibit SIV and HIV-1 replication *in vitro* (50–52). However, the inhibitory effects of cyclosporine were not a factor for our challenge, as these macaques were challenged 11 days after the cessation of CsA treatment (5.5 weeks after AAV injection), and all macaques given control AAV8 vectors after cyclosporine treatment became infected (Fig. 6B).

Inhibitory antibodies developed against the transgenic antibody simVRC07 in macaques in the current study, despite our efforts to simianize the antibody. In our previous study of passive protein transfer, human VRC01 was immunogenic in two macaques given a single 20-mg/kg dose, but simian VRC01 was not (31). The development of antibodies against simVRC07 was not entirely unexpected, as it contains all three huVRC07 CDRs in their entirety and 24 human residues within its framework regions. The development of antibodies against huVRC07 in humans would be unexpected, as most human antibodies, when delivered by passive protein transfer, are not immunogenic (53). However, for comparison to our previous studies with simian VRC01, we passively transferred 5 mg/kg of simian VRC07 protein into two macaques and did not observe any detectable anti-simian VRC07 plasma antibody responses. Therefore, passive transfer of antibody protein may not be predictive of the immunogenicity of AAV-delivered antibodies. Although we generally have observed more anti-antibody responses after administration of the vector than after administration of protein, we cannot currently draw definitive conclusions about the relative immunogenicity of each modality. Future studies will help to define the relative contribution of each modality. Vectored gene delivery of a human antibody in humans has yet to be reported; therefore, whether antitransgene antibodies will be observed remains to be determined.

The antitransgene antibodies observed here were inhibitory but more importantly were correlated inversely with simVRC07 plasma levels (Fig. 3B). This inverse correlation suggested that these antibodies were binding to circulating simVRC07 IgG in macaques and contributed to its clearance. Thus, antitransgene antibody responses represent one factor that is predictive of the failure of AAV-vectored antibody gene transfer. We show here that transient immunosuppressive monotherapy is sufficient to increase the longevity of transgene expression in most cases. These results corroborate data from previous studies that used immune suppression to eliminate anti-factor IX antibodies in canines and macaques after AAV delivery (33, 54). Reduction of transgene immunogenicity has also been achieved by reducing the vector dose at single or multiple injection sites (54–56). It is possible that the two doses tested here were still above the threshold for reducing transgene immunogenicity and that administering a very low dose of the vector may still reduce transgene immunogenicity. However, lowering the dose of the vector to prevent transgene immunogenicity will have to be balanced with the need to generate protective concentrations of the transgenic antibody.

The molecular mechanisms that resulted in persistent expression of the HIV-1 bnAb in nonhuman primates after cessation of immune suppression are presently unclear. One hypothesis is an induction of immune tolerance to the transgenic antibody. B cells attempting to respond to simVRC07 during immune suppression would not receive the T cell help that is required according to the two-signal activation model (57). These B cells would subsequently be clonally deleted or rendered anergic, reducing the antitransgene humoral response once immune suppression is halted. Alternatively, the antigen persistence model could explain the induction of tolerance in our system (58). According to this model, the persistence of simVRC07 in secondary lymphoid organs over the 4-week immunosuppression period would lead to tolerance that prevents the development of anti-simVRC07 antibodies after withdrawal of immune suppression.

The global health community awaits a protective intervention to curb the spread of the HIV-1 pandemic in at-risk populations. AAV-vectored bnAb gene transfer can protect humanized mice from HIV-1 infection and may be able to similarly create a sustained protective barrier in humans. In support of this notion, we show here that gene transfer of a bnAb can protect macaques from mucosal SHIV infection >1 month after vector delivery. These results extend the protective efficacy seen previously in humanized mice (21) to SHIV infection in nonhuman primates and provides support for further investigation in humans.

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