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Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys

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The key to an effective HIV vaccine is development of an immunogen that elicits persisting antibodies with broad neutralizing activity against field strains of the virus. Unfortunately, very little progress has been made in finding or designing such immunogens. Using the simian immunodeficiency virus (SIV) model, we have taken a markedly different approach: delivery to muscle of an adeno-associated virus gene transfer vector expressing antibodies or antibody-like immunoadhesins having predetermined SIV specificity. With this approach, SIV-specific molecules are endogenously synthesized in myofibers and passively distributed to the circulatory system. Using such an approach in monkeys, we have now generated long-lasting neutralizing activity in serum and have observed complete protection against intravenous challenge with virulent SIV. In essence, this strategy bypasses the adaptive immune system and holds considerable promise as a unique approach to an effective HIV vaccine.

Development of an HIV vaccine is proving to be a daunting task. In the 25 years since the discovery of HIV, hundreds of vaccine candidates have been vetted in a variety of animal models. Many have also been tested in early-phase human clinical trials with mostly disappointing results. Two vaccine approaches, each targeting a different arm of the adaptive immune response, have been evaluated in large efficacy trials. Both failed to protect vaccine recipients from infection, and neither diminished viral replication after infection[1–4]. Although there are other candidates in the pipeline, it seems unlikely that a noteworthy breakthrough is imminent.

These sobering observations underscore the tremendous hurdles that must be overcome to develop an effective HIV vaccine[5–9]. Foremost among these hurdles is the inability to induce antibodies that neutralize a wide array of HIV field isolates. Such antibodies are rarely found in the sera of long-term–infected humans[10,11], and only a handful of broadly neutralizing human monoclonal antibodies have been isolated[12–15]. Therefore, one can conclude that broadly neutralizing antibodies are both relatively rare and difficult to elicit, even after acute and chronic natural infection. It seems unlikely that vaccine developers will improve upon natural responses with contrived immunogens, at least in the near term.

Passive immunization schemes using neutralizing antibodies have protected monkeys from SIV or simian-human immunodeficiency virus (SHIV) challenge infections[16–18]. Unfortunately, an injection of antibodies every few weeks is neither practical nor cost effective as a large-scale human vaccine approach. Some years ago, it was shown that sustained delivery of antibodies could be achieved in mice by implanting collagen-encapsulated 3T3 fibroblasts that had been transduced *ex vivo* with a retroviral vector carrying an antibody gene[19]. HIV-specific antibodies that seeped out of the implant decreased viral burden in HIV-1–infected, humanized immunodeficient mice.

Our interest in adeno-associated virus (AAV) vectors caused us to consider such vectors as a means to deliver HIV-specific antibodies directly to muscle, thereby avoiding *ex vivo* manipulations. In this scheme, the antibody gene of choice is packaged into an AAV vector, which is then delivered by direct intramuscular injection. Thereafter, antibody molecules are endogenously synthesized in myofibers and passively distributed to the circulatory system. In a proof-of-concept experiment, mice injected with an AAV vector carrying the gene encoding IgG1b12 produced authentic, biologically active IgG1b12 that was detected in mouse sera for over 6 months[20].

These data, although encouraging, were generated in mice, which is an artificial model system that is sometimes difficult to translate to higher-order primates. Moreover, it was unclear whether the serum levels of neutralizing activity generated would translate to protection from a challenge infection. To more rigorously test the basic concept of antibody gene transfer as an approach to immunization, we turned to the well characterized SIV model system in monkeys. Here we describe the adaptation of the original antibody gene transfer concept to macaques and also detail the derivation of macaque-specific 'designer' molecules (immunoadhesins) that neutralize SIV and afford protection from SIV challenge infection.

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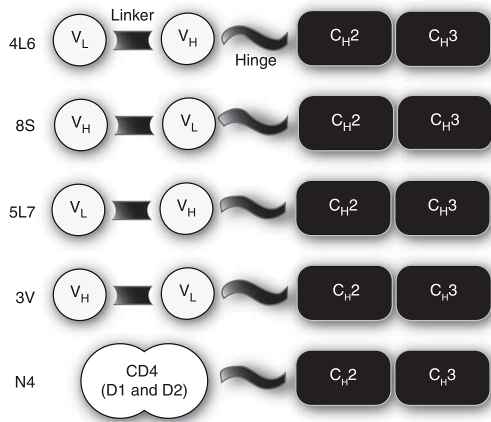

Figure 1 Schematic representation of immunoadhesin constructs. For molecules derived from macaque Fabs (4L6, 8S, 5L7 and 3V), V_H and V_L domains were joined by a synthetic linker. Rhesus CD4 (domains 1 and 2 (D1 and D2)) was cloned as described in the Methods online. Antigen-binding domains were attached to the Fc fragment of a rhesus IgG2 molecule.

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RESULTS

SIV immunoadhesins

In pilot experiments in mice (J.Z., B.C.S., P.R.J. and K.R.C., unpublished data), we showed that immunoadhesins (defined as chimeric, antibody-like molecules that combine the functional domain of a binding protein with immunoglobulin constant domains) were superior to single-chain (scFv) or whole-antibody (IgG) molecules with respect to achievable steady-state serum concentrations. Our constructs followed a formula whereby an antiviral moiety was fused to an IgG Fc domain (**Fig. 1**).

To generate SIV-specific immunoadhesins with native macaque sequences, we obtained previously characterized SIV gp120-specific Fab molecular clones that had been derived directly from SIV-infected macaques[21]. These Fab molecules were shown to neutralize several defined stocks of SIV *in vitro*, thereby affording us the opportunity to test *in vivo* neutralizing activity after gene transfer to macaques. For these experiments, we chose two Fabs for further modification: 346-16h and 347-23h (ref. 21). Both Fabs had potent *in vitro* neutralizing activity against SIVmac316, a macrophage-tropic derivative of SIVmac239 (refs. 21–23). Consequently, we chose SIVmac316 as our challenge strain. Although SIVmac316 has not routinely been used in SIV vaccine experiments, it was ideally suited for our purposes because we were able to match antibody reagents with a pathogenic SIV strain. In the end, the SIVmac316 challenge stock was highly infectious and pathogenic for rhesus monkeys (see below).

We joined the variable heavy (V_H) and variable light (V_L) chains from the Fabs via a linker to create an scFv and then joined the scFv to a rhesus IgG2 Fc fragment (**Fig. 1**). Because the linear order of the V_H and V_L moieties was arbitrary, we made both orientations for each Fab. We also included a construct based on the rhesus CD4 domains 1 and 2, modeled after CD4-γ fusion proteins[24].

When we transfected the plasmid into 293 cells, the expressed proteins were secreted into the cell culture medium as disulfide-linked homodimers (data not shown). They all bound SIV gp120 in a standard ELISA, and each construct showed substantial *in vitro* neutralizing activity against SIVmac316 (**Fig. 2**). We subsequently chose constructs 4L6 and 5L7 (representing Fabs 346-16h and 347-23h, respectively) for testing in macaques. We also chose to test N4 because of its unique composition, despite the fact that it did not neutralize as potently as 4L6 or 5L7.

We individually cloned expression cassettes for 4L6 and 5L7 between AAV inverted terminal repeats designed to produce self-complementary genomes[25]. Such genomes have been shown to direct more efficient *in vitro* and *in vivo* transduction when compared to traditional single-stranded DNA AAV vectors[26]. N4 is a traditional single-stranded DNA genome because we made it before the self-complementary vector was available to us. We packaged all three recombinant genomes into AAV serotype 1 capsids by standard techniques[27].

Immunization by *in vivo* gene transfer

We immunized nine rhesus macaques with AAV vectors carrying immunoadhesin constructs: three received 4L6, three received 5L7 and three received N4. We administered each vector at time 0 by intramuscular injection (2×10^{13} vector genomes), and we periodically collected serum from each macaque and tested it for protein abundance and biologic activity. Immunoadhesin concentrations in the 4L6 vector recipients reached 100–190 μg ml^{-1} by 4 weeks after immunization, which was the time of challenge (**Fig. 3a**). These concentrations corresponded well with measured serum neutralization titers on the day of challenge ranging from 1 in 2,560 to 1 in 5,120 (**Fig. 2b**).

Two 5L7-immunized monkeys (05C002 and 05C053) had a prechallenge course similar to the 4L6-immunized monkeys. Serum concentrations of the immunoadhesin reached 40 μg ml^{-1} and 175 μg ml^{-1}, respectively, by the day of challenge (**Fig. 3a**), which corresponded to measured serum neutralization titers of 1 in 1,280 and 1 in 20,480 (**Table 1**). The third 5L7 recipient (05C004) was different. The 5L7 immunoadhesin was present at 2 weeks after immunization (50 μg ml^{-1}), but was undetectable by 4 weeks after immunization (**Fig. 3b**). This rise and fall was reflected in serum neutralization titers that were 1 in 3,840 at 2 weeks and $<$1 in 32 at 4 weeks after immunization (**Table 1**). This decline reflected the appearance of 5L7-specific antibodies in this macaque (details below).

The three macaques immunized with N4 had much lower serum concentrations of immunoadhesin on the day of challenge (3–10 μg ml^{-1}; **Fig. 3c**) when compared to 4L6-immunized monkeys. Serum neutralizing titers were likewise lower in these macaques, ranging from 1 in 64 to 1 in 128 (**Table 1**). The lower titers are probably due to the fact that N4 is a single-stranded DNA vector, resulting in less efficient vector transduction[26].

SIV challenge

Four weeks after immunization, we gave all nine macaques an intravenous injection of SIVmac316. In addition, at that time we

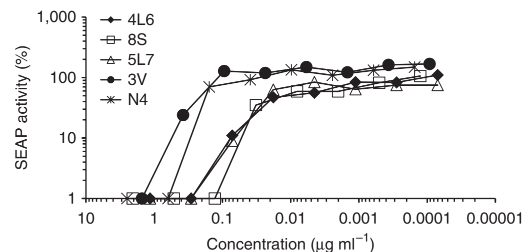

Figure 2 Neutralization of SIV *in vitro*. *In vitro* neutralizing activity against SVmac316 of the five immunoadhesins depicted in **Figure 1**. All five showed substantial 50% neutralization at $<$1 μg ml^{-1}. The half-maximal inhibitory concentration (μg ml^{-1}) for each was 4L6 (0.01), 8S (0.01), 5L7 (0.02), 3V (0.20) and N4 (0.25). SEAP, secreted alkaline phosphatase.

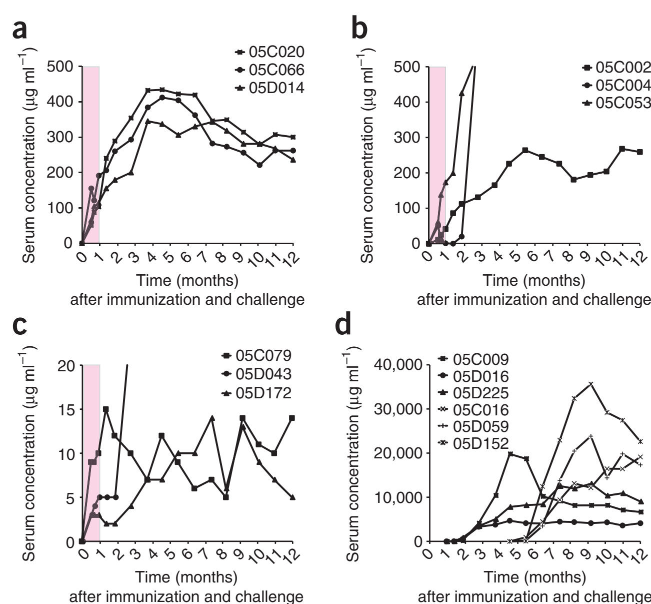

Figure 3 Serum concentration of immunoadhesins or antibodies after gene transfer and SIV challenge. (**a**–**d**) Serum concentrations of immunoadhesins or antibodies in 4L6 immunized macaques (**a**), 5L7-immunized macaques (**b**), N4-immunized macaques (**c**) and unimmunized controls infected at two different times (1 month and 4.5 months) (**d**). Sera from unimmunized and immunized macaques were tested over time by a SIV gp120 ELISA for immunoadhesins (or antibodies in infected macaques) after immunization and challenge infection (see Methods online). The day of challenge was 1 month after immunization. In **b**, the reactivity to gp120 for monkeys 05C004 and 05C053 was off scale (owing to SIV infection) and peaked at 5,023 and 1,191 μg ml^{-1}, respectively. In **c**, the reactivity to gp120 for monkey 05D043 was off scale (owing to SIV infection) and peaked at 3,953 μg ml^{-1}. The pink shaded areas in **a**–**c** represent the time period after immunization and before challenge.

injected three unimmunized control macaques with the same dose of the same challenge stock. Sixteen weeks later, we inoculated three more unimmunized control macaques with the same dose of the same challenge stock (for a total of six naive control macaques). Viral loads after challenge are shown in **Figure 4**.

The challenge virus infected all six unimmunized control macaques, with peak plasma viral loads ranging between $1 \times 10^{6.5}$ to 1×10^{8} RNA copies ml^{-1} (**Fig. 4d**). Only a single control macaque (05D016) suppressed viral replication to below 1×10^{5} copies ml^{-1} in the post-acute phase of infection. In fact, we had to kill four of the six macaques between 57 and 60 weeks after infection, owing to AIDS-related complications. With death as an endpoint, the immunized macaques were significantly protected (zero of nine died) relative to the unimmunized controls (four of six died; $P = 0.01$).

In contrast to the control monkeys, six of the nine immunized macaques remained uninfected after challenge, as judged by a lack of SIV RNA in plasma (**Fig. 4a–c**) and lack of antibodies to Gag (**Supplementary Fig. 1** online). All three of the 4L6 macaques were protected from infection (compared to unimmunized controls, $P = 0.01$), while two of three were protected in the N4 group ($P = 0.08$), and one of three in the 5L7 group ($P = 0.33$).

Humoral responses after SIV challenge

As expected, all six naive control monkeys had SIV-specific antibody responses after infection. All developed antibodies to gp120 (**Fig. 3d**) and Gag (**Supplementary Fig. 1**). There was also a concomitant rise in serum neutralizing activity. By 8 weeks after challenge, neutralization titers in the three naive controls ranged from 1 in 20,480 to 1 in 81,920 (**Table 1**). We did not test the three control macaques (05C016, 05D059 and 05D152) infected at 16 weeks after the first group for neutralizing antibodies before or after challenge.

Likewise, the three immunized macaques that became infected after challenge (05C053, 05C004 and 05D043) also developed antibodies to gp120 and Gag (**Supplementary Fig. 1**). Shortly after challenge, each had sizable increases in gp120-specific antibodies detectable by ELISA that were clearly distinguishable from those generated by AAV vector–mediated gene transfer (**Fig. 3b,c**). These higher levels also correlated with increases in serum neutralization titers. By 8 weeks after challenge, titers in these monkeys ranged from 1 in 40,960 to 1 in 81,920 (**Table 1**).

The six immunized macaques that remained uninfected after challenge had a distinctly different profile. All three monkeys that received 4L6 were protected from challenge infection and had serum neutralizing activity on the day of challenge that was maintained through 8 weeks after challenge (**Table 1**). Serum 4L6 concentrations gradually rose to a peak at about 4 months after immunization and ultimately plateaued between 8 and 12 months at 200–300 μg ml^{-1} (**Fig. 3a**). 5L7 recipient 05C002 was also protected from infection and had a pattern of immunoadhesin abundance (**Fig. 3b**) and neutralizing activity (**Table 1**) similar to the 4L6 monkeys. This general pattern of expression was consistent with the biology of AAV vectors and has been shown for other transgene products in mice, dogs and monkeys[28–30]. Moreover, unlike antibodies that arise in response to SIV infection, but like the authentic immunoadhesins, the gp120-binding and neutralizing activity in the sera of these monkeys at 9 months after immunization was completely sensitive to heat treatment at 56° for 30 min (data not shown)[31].

Two N4 monkeys were also protected (05C079 and 05D172), even though serum neutralization titers on the day of challenge were much lower than in the other four protected monkeys (**Table 1**). As with the other protected monkeys, serum concentrations of N4 were maintained through 12 months, albeit at much smaller concentrations (5–15 μg ml^{-1}).

Table 1 Serum neutralizing activity against SVmac316 before and after immunization and challenge

		Time (weeks) after immunization				
Vaccine	Macaque	0	2	4 (DOC)	6	12
None	05C009	<32	<32	<32	<32	20,480
	05D016	<32	<32	<32	<32	20,480
	05D225	<32	<32	<32	<32	81,920
4L6	05C020	<32	640	2,560	2,560	5,120
	05C066	<32	5,120	5,120	2,560	5,120
	05D014	<32	640	2,560	2,560	5,120
5L7	05C002	<32	480	1,280	2,560	5,120
	05C004	<32	3,840	<32	<32	40,960
	05C053	<32	1,920	20,480	30,720	40,960
N4	05C079	<32	128	128	256	128
	05D043	<32	64	128	64	81,920
	05D172	<32	64	64	64	64

Titers shown are reciprocal serum dilutions representing 50% neutralization. The day of challenge (DOC) was at week 4.

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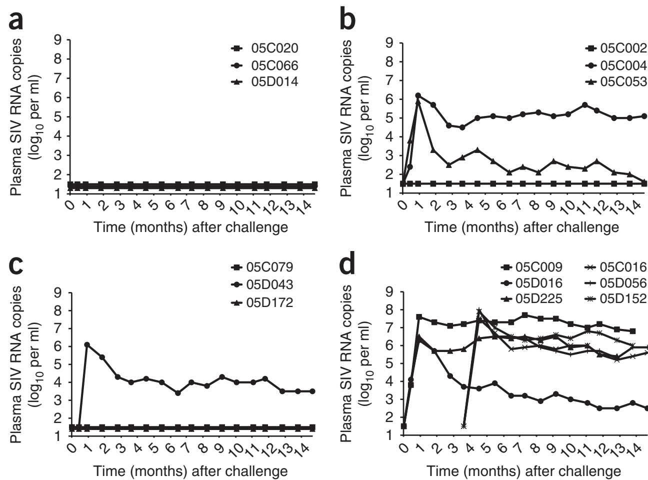

Figure 4 Detection of SIV in plasma after challenge. (**a**–**d**) SIV viral loads after challenge infection of 4L6-immunized macaques (**a**), 5L7-immunized macaques (**b**), N4-immunized macaques (**c**) and unimmunized controls (**d**). Plasma samples from unimmunized and immunized macaques were tested after challenge infection for SIV RNA genomes (viral load). The day of challenge was 0 months (and 3.5 months for the second set of unimmunized control macaques). Monkeys C009, D225, D059 and D152 from the unimmunized control group were killed between 57 and 60 weeks after infection, owing to AIDS-related complications.

Correlates of protection

Variations in protection among immunized macaques allowed us the opportunity to search for potential correlates of protection. A clue came from macaque 05C004, who had an early peak and then a rapid decline in serum 5L7, such that by the day of challenge, neutralizing activity in the serum was below the threshold of detection (**Table 1**). This scenario was similar to expression patterns observed in mice with transgene-specific antibody responses (J.Z., B.C.S., P.R.J. and K.R.C., unpublished data). To explore this possibility, we used purified 5L7 protein as the solid-phase antigen and tested 05C004 serum in a standard ELISA (**Fig. 5a**). The reactivity pattern showed a high-titered 5L7-specific antibody response that was detectable by 3 weeks after immunization, peaked at 8 weeks and declined thereafter (**Fig. 5a**). These data are consistent with the peak and rapid decline of the 5L7 immunoadhesin in the serum of macaque 05C004, and they also explain why the macaque became infected.

We next tested serum from the rest of the immunized macaques in the same fashion (**Fig. 5a**). It was readily apparent that the other two immunized monkeys that became infected (05C053 and 05D043) also had immunoadhesin-specific antibody responses, albeit at much lower levels than 05C004 (**Fig. 5a**). None of the protected monkeys showed immunoadhesin-specific responses (**Fig. 5a**).

In contrast to macaque 05C004, macaques 05C053 and 05D043 possessed *in vitro* serum neutralizing activity on the day of challenge (**Table 1**). In fact, macaque 05C053 had the highest serum neutralization titer of any of the macaques (1 in 20,480) on the day of challenge (**Table 1**).

These data prompted us to examine the specificity of the immunoadhesin-specific responses. Antibodies from each macaque had a distinct specificity (**Fig. 5b**). Serum from 05C004 (5L7 recipient) reacted with purified 5L7 and 4L6, but not N4, suggesting that these antibodies were raised against the framework sequence in the scFv domain of 5L7 and 4L6. Serum from 05C053 (5L7 recipient) reacted with all three immunoadhesins, suggesting that these antibodies were raised against the Fc fragment that each chimeric protein had in common. Finally, serum from 05D043 (N4 recipient) reacted only with N4, indicating specificity for the CD4 moiety that is unique to N4.

DISCUSSION

In the experiments described here, we used gene transfer technology to effectively bypass the adaptive immune system to generate long-lived, protective anti-SIV biological activity in the sera of otherwise naive monkeys. Six of nine immunized monkeys were protected against infection by the SIV challenge, and all nine were protected from AIDS, whereas all six of the controls became infected and two-thirds (four of six) died over the course of the experiment.

Much of the success in these experiments can be attributed to AAV gene transfer technology. AAV vectors have an established record of high-efficiency gene transfer in a variety of model systems[32,33]. When delivered to post-mitotic organs such as muscle, brain and liver, AAV vector genomes take on the form of intranuclear high–molecular weight episomal concatamers that direct transgene expression for extended periods of time[34–39]. Our previous work demonstrated the feasibility of this approach in mice for *in vivo* HIV neutralization[20], and other investigators have adapted AAV (and other vector systems) for antibody gene delivery for a variety of purposes[40–50]. Here we showed that in monkeys a single intramuscular injection of an AAV vector could direct long-term (>1 year) continuous expression of a biologically active protein. In addition, the serum immunoadhesin concentrations achieved with the self-complementary (double-stranded DNA) AAV vectors (4L6 and 5L7) were far superior to those observed with the traditional single-stranded DNA vector (N4).

Three key caveats emerged from our data. First, transgene immunogenicity seemed to be a major correlate of protection that must be better understood. We tested three different immunoadhesins and observed immunoadhesin-specific responses that ran the gamut from

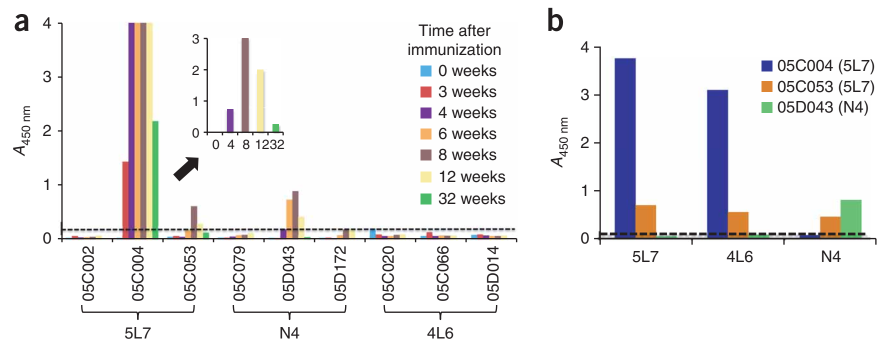

Figure 5 Immunoadhesin antibodies in immunized monkeys. (**a**) Reactivity of serum (1 in 100 dilution) collected over time from each immunized macaque for immunoadhesins, as tested by ELISA. For example, sera from 5L7 recipients 05C002, 05C004 and 05C053 were reacted against purified 5L7 protein (as indicated on the *x* axis). The dashed line indicates the limit of sensitivity. The inset shows sera from macaque 05C004 at a 1 in 1,000 dilution. (**b**) Reactivity of sera from the three indicated macaques for the respective purified proteins (as indicated on the *x* axis). As expected, each macaque serum reacted with the homologous immunoadhesin. 05C004 reacted against 5L7 (homologous) and 4L6 (heterologous). The dashed line indicates the limit of sensitivity.

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undetectable (4L6) to almost completely incapacitating (5L7). It is tempting to speculate, but remains unproven, whether immunoadhesins expressed by *in vivo* transduction might behave like exogenously administered recombinant DNA–derived proteins (for example, monoclonal antibodies or chimeric molecules such as etanercept) whose safety profiles can be readily established. Moreover, it should be possible to lower the potential for immunogenicity by specific residue modifications[51,52]. Notably, none of the three macaques who showed immunoadhesin-specific antibody responses has shown clinical signs or symptoms of disease.

A second caveat is that traditional *in vitro* neutralization assays did not seem to faithfully represent *in vivo* activity. Although macaques without measurable neutralizing activity on the day of challenge were all infected, the opposite was not true; the presence of *in vitro* neutralizing activity, even at high titer, did not guarantee protection. Macaque 05C053 showed an immunoadhesin-specific antibody response, directed at the Fc domain of the chimeric protein, that did not inhibit *in vitro* neutralizing activity. In fact, this macaque had the highest neutralizing titer of all of the immunized macaques on the day of challenge (**Table 1**). These data suggest that immunoglobulin effector functions not measured in standard *in vitro* assays (such as Fc activity) might be crucial for *in vivo* neutralizing activity[53]. Notably, our data seem to confirm recent work in which the Fc effector function of a neutralizing monoclonal antibody was inactivated by mutation, and the *in vivo* protective effect of the antibody was blunted[54]. These observations suggest that optimizing antibody functions over and above antigen binding might be of benefit in future iterations of antiviral transgenes.

A final caveat is that we used the intravenous route for the SIV challenge. Because most new HIV infections occur across a mucosal surface, it will be crucial to show protection against a mucosal challenge. However, there is cause for optimism. Several studies have shown that traditional systemic passive immunization with antibody preparations can protect against an SIV or SHIV mucosal challenge[16–18]. Moreover, there is ample evidence to suggest that systemic immunization with a nonreplicating immunogen can protect from natural infection at a mucosal surface. A recent example is the vaccine against human papillomavirus, which is a nonreplicating virus-like particle that, when given intramuscularly, elicits antibodies that protect against infection of the female genital tract[55]. Notably, the macaques in the current study immunized by gene transfer maintained high levels of circulating antibodies that probably permeated most body tissues and mucosal surfaces.

Although considerable hurdles remain, the HIV immunization approach outlined here seems to be a viable alternative to more traditional strategies. To ultimately succeed, more and better neutralizing monoclonal antibodies against primary HIV isolates that can be tested in gene transfer experiments will be needed. Also, more nonantibody inhibitors such as CD4 and its derivatives should be developed in anticipation of combinatorial approaches that target multiple steps along the HIV entry pathway. And, finally, as we have shown here, the SIV-monkey model can be used to investigate a variety of variations on this theme.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

Project planning was performed by P.R.J., B.C.S. and K.R.C.; experimental work was performed by B.C.S., J.Z., M.J.C., S.M.G. and E.Y.; data analysis was performed by P.R.J., K.R.C., R.C.D., B.C.S., J.Z., M.J.C. and S.M.G.; and the manuscript was composed by P.R.J., R.C.D., B.C.S. and K.R.C.

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ONLINE METHODS

Simian immunodeficiency virus–specific immunoadhesin gene constructs. We synthesized DNA (Geneart) with optimized codons. We used SIV Fab[21] 346-16h to derive 4L6 and 8S, and we used Fab 346-16h to derive 5L7 and 3V. We joined the variable domains by a 15–amino acid glycine-serine $(G4S)_3$ linker, and we placed a synthetic signal peptide for optimized secretion[56] at the 5′ end. The Fc fragment was rhesus IgG2 cloned from lymphocyte RNA. We placed each construct between a cytomegalovirus promoter (similar to one previously published[57]) and a synthetic polyadenylation signal[58]. N4 contained the rhesus CD4 signal sequence followed by the D1 and D2 domains of rhesus CD4.

Recombinant proteins. We transfected (Superfect, Qiagen) HeLa cells (American Type Culture Collection) with plasmid, and we purified proteins from the medium using protein-A (Nunc International). We quantified purified proteins by ELISA, using purified rhesus IgG as a standard (Bethyl Laboratories).

Adeno-associated viral vectors. We produced and purified all vectors as previously described[25,27,59]. Titers ranged between 2×10^{12} and 1×10^{13} vector genomes per ml.

Monkeys and immunization. We purchased rhesus macaques of Indian origin from Covance and housed them in the vivarium at the Research Institute at Nationwide Children's Hospital in accordance with standards set forth by the American Association for Accreditation of Laboratory Animal Care. All macaques tested negative for antibodies to SIV, simian type D retrovirus and simian T-cell lymphotropic virus type 1. Their weights at the time of immunization ranged from 2.7 kg to 4.2 kg. We gave each immunized macaque 2×10^{13} vector genomes divided into four equal portions (0.75 ml each), delivered by four separate (two in each quadriceps) deep intramuscular injections.

Simian immunodeficiency virus neutralization assay. We tested purified proteins or macaque sera for *in vitro* neutralization activity with the previously described secreted alkaline phosphatase assay[22].

Immunoadhesin concentrations. We measured 4L6, 5L7 and N4 concentrations in serum by using SIV gp120 to coat ELISA plates (100 ng per well) in PBS buffer at 4 °C overnight. We decanted the antigen and blocked the wells with 3% BSA in PBST (PBS + 0.1% Tween-20) for 2 h with constant shaking at 25 °C. After washing with PBST, we incubated the wells with 100 μl of diluted serum (or 4L6, 5L7 or N4 standards) for 1 h at 25 °C. After washing, we added horseradish peroxidase–conjugated secondary goat antibody to human IgG-Fc HRP (Bethyl Laboratories; 1 in 1,000) to each well (100 μl) for 30 min at 25 °C. After four more washes, we added tetramethylbenzidine substrate (100 μl, Pierce) for 10 min at 25 °C. After we terminated the reaction with 1 N H_2SO_4, we read the plates at an absorbance at 450 nm on a Molecular Devices microplate spectrophotometer. We quantified the results by extrapolation from the standard curve with software from Molecular Devices (coefficient of linearity ≥ 0.99). The assay sensitivity was 0.4 ng ml^{-1} for 5L7 and 4L6 and 4 ng ml^{-1} for N4.

Transgene-specific antibody responses. We coated plates with purified N4, 5L7 or 4L6 purified proteins in PBS buffer (100 ng per well) overnight at 4 °C and processed them as described above. We added mouse antibody to human IgG1 (1 in 500 dilution; Sigma-Aldrich) in blocking buffer to the wells and incubated them at 25 °C for 30 min. We chose an IgG1 isotype–specific secondary antibody to avoid cross-reactivity with the IgG2 immunoadhesins. After washing, we incubated the wells with 100 μl (1 in 1,000 dilution) of a rabbit mouse IgG-Fc–specific horseradish peroxidase conjugate (Sigma-Aldrich) for 30 min. We performed TMB substrate development as described above. To detect cross-reactivity in specific monkey serum samples, we processed alternative coating antigens in an identical manner as that used for the cognate coating antigen.

Simian immunodeficiency virus challenge. We generated infectious SIVmac316 by transfecting full-length proviral DNA into 293T cells (American Type Culture Collection) by the calcium phosphate method (Promega). We measured virus concentrations in the supernatant by determining the concentration of p27 capsid protein using an antigen-capture assay according to the manufacturer's instructions (SIV p27 Antigen Capture Assay, Advanced Bioscience Laboratories). The virus stock used in this study contained 357 ng ml^{-1} of p27. We diluted the virus stock 1 in 2,500 in sterile PBS and injected1 ml intravenously. We estimated that 1 ml of the diluted stock contained approximately 40 macaque infectious doses by comparison of its infectivity in cell culture to that of other SIV stocks. We determined viral loads in plasma with a quantitative real-time RT-PCR assay as previously described[60].

Statistical analyses. Because of small sample sizes, we used Fisher's exact probability test (two-tailed) to examine the differences between groups.

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