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Original Article

Protection against SIV in Rhesus Macaques Using Albumin and CD4-Based Vector-Mediated Gene Transfer

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Antibody-like molecules were evaluated with potent simian immunodeficiency virus (SIV) neutralizing properties (immunoadhesins) that were delivered by a recombinant adeno-associated virus (rAAV) vector in the SIV-infected rhesus macaque model. When injected intramuscularly into the host, the vector directs *in vivo* production of the transgenes with antibody-like binding properties that lead to serum neutralizing activity against SIV. To extend the half-life of the immunoadhesins, rhesus cluster of differentiation 4 (CD4) and a single-chain antibody (4L6) were fused with albumin molecules, and these constructs were tested in our model of SIV infection. Antibody-based immunoadhesins provided high serum neutralizing titers against the original SIV strain. CD4-based immunoadhesins provided a wider spectrum of neutralization against different SIV strains in comparison to antibody-based therapeutics and had the potential to protect against high viral challenging doses. Although the albumin-antibody fusion immunoadhesin provided strong and prolonged protection of the immunized animals against SIV challenge, the albumin-CD4 fusion altered the specificity and decreased the overall protection effectiveness of the immunoadhesin in comparison to the antibody-based molecules. Albumin-based immunoadhesins increase *in vivo* longevity of the immune protection; however, they present challenges likely linked to the induction of anti-immunoadhesin antibodies.

INTRODUCTION

Approaches toward a human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) vaccine require the induction of antibodies that neutralize a wide array of HIV/SIV field isolates.[1,2] Such antibodies are rare; nevertheless, over the past few years, several HIV antibodies have been identified with a broad range of viral neutralization and high antibody potency.[3–6] Newer antibodies were isolated by high-throughput screening of sera from HIV-1-infected individuals, categorized as "elite neutralizers," based on their neutralization breadth and potency.[7,8] Extensive sequence analysis of these potent, broadly neutralizing antibodies revealed that high levels of somatic mutations were required to generate mature antibodies.[9] Furthermore, the maturation process may involve repeated rounds of antibody selection through HIV- antigen interaction. Taken together, these observations indicate that the induction of potent, broadly neutralizing antibodies using traditional vaccine strategies (e.g., subunit proteins or viral vectors) remains a major challenge.

Passive immunization using neutralizing monoclonal antibodies has protected rhesus macaques from SIV/HIV (SHIV) challenge infections.[10–12] Injection of antibodies every few weeks is not practical or cost effective compared to a large-scale prophylactic vaccine approach. Another option is to isolate the representative antibody gene and use gene-transfer technology to endow a target host with the gene. In this system, the antibody gene directs endogenous expression of the antibody molecule, and the host produces circulating antibodies.[13] The HIV/SIV antibody gene is packaged into a recombinant adeno-associated virus (rAAV) vector, which is delivered by direct intramuscular injection. Thereafter, antibody molecules are endogenously synthesized in myofibers and passively distributed to the circulatory system.[8] This approach has been used in many nonhuman primate and murine models[14–23] and ongoing studies in the field of HIV/SIV. In contrast to antibodies, immunoadhesins are engineered molecules containing antigen-binding domains, as well parts designed to improve overall performance, such as to increase *in vivo* half-life and to decrease immunogenicity.[24] In a proof-of-concept study by Johnson et al., rhesus macaques were injected with rAAV vectors expressing monkey antibody and cluster of differentiation 4 (CD4)-based fusion proteins (immunoadhesins) that neutralized *in vitro* SIV.[24] Monkeys were protected from infection following challenge with virulent SIV, and neutralizing antibodies were detected in monkey sera for over 4 years following a

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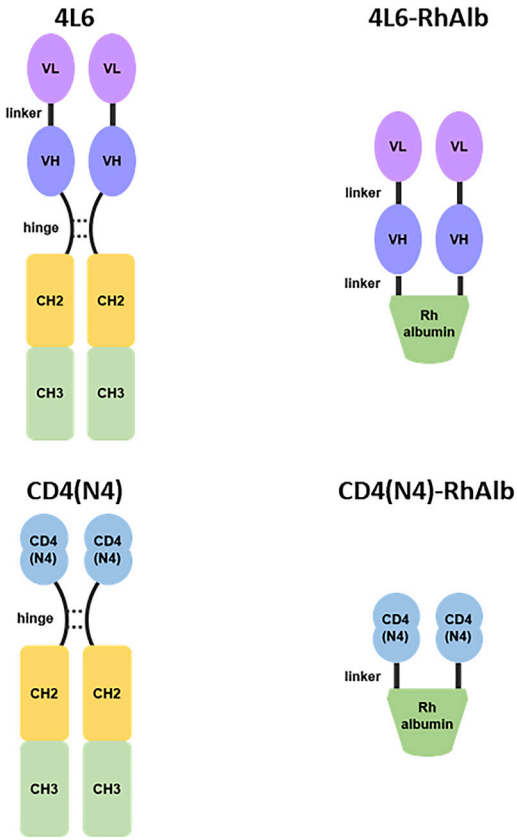

Figure 1. Schematic Representation of the 4 Immunoadhesin Constructs Used in the Current Study

Schematic representation of the immunoadhesin constructs. 4L6 antibody-based constructs containing the Fc fragment of rhesus IgG2 or rhesus albumin are identified as "4L6" and "4L6-RhAlb," respectively. The CD4 construct is identified as "CD4(N4)." The CD4-Albumin-CD4 construct is identified as "CD4(N4)-RhAlb." Fab 4L6, variable heavy-chain (VH), and variable light-chain (VL) domains, was joined by a $(G_2S)_3$ synthetic linker. Combined 4L6 was joined to rhesus albumin by a $(G_2S)_4$ synthetic linker. For further details, see Materials and Methods.

single intramuscular injection.[24] In human studies, utilization of rAAV-based technology in order to express HIV neutralizing antibodies in a native, full-length format was well tolerated, and although it yielded very low levels of circulating antibodies,[25] it suggests that the immunoadhesin format may be a viable therapeutic approach.

We have built on the work to improve *in vivo* efficacy and longevity of immunoadhesin constructs. Albumin is the molecule of choice for a fusion partner, due to its long half-life, safety profile, and low potential for immunogenicity. It is the most abundant protein in plasma and is ubiquitous at mucosal surfaces.[26,27] Albumin has a half-life of approximately 20 days in serum,[28] lacks enzymatic function, and has a low potential for immunogenic reactions.[29] Albumin fusion constructs increase the half-life of smaller partners, such as single-chain antibodies. Albumin-fusion products have been tested in humans, including albumin-interferon fusion, albumin-coagulation factor IX fusion, and albiglutide (albumin-GLP-1 peptide for the treatment of diabetes).[30–34] Compounds containing albumin fused with HIV inhibitors have also been created. Soluble CD4 (sCD4), which contains the CD4 domains 1 and 2, was fused directly to the C terminus of albumin, resulting in a dramatic increase in half-life *in vivo* compared to free sCD4, without loss in gp120 binding.[35] An albumin protein with a chemically conjugated C34 peptide (fusion inhibitor) was evaluated as an HIV therapeutic.[36,37] Albumin fusion significantly decreases renal clearance and degradation of peptides. For instance, an albumin-C34-conjugated protein injected directly into mice resulted in an increase in C34 half-life with prolonged antiviral activity, indicating that less frequent dosing would be needed compared to an unconjugated peptide.[37] Since albumin fusion can increase the *in vivo* longevity of the resulting protein, without interfering significantly with its activity, we generated and tested a novel class of HIV inhibitors based on albumin fusion proteins. The extension of the *in vivo* half-life of the immunoadhesins would translate into a more practical and cost-effective approach for HIV prophylaxis.

RESULTS

We utilized the 4L6 and CD4(N4) immunoadhesins, which were tested in our previous study, and performed Fc fusion with either a single-chain antibody or rhesus CD4. The 4 immunoadhesins, 2 from Johnson et al.[24] and 2 new, rhesus albumin (RhAlb) fusion-based constructs 4L6-RhAlb and CD4(N4)-RhAlb, are summarized in Figure 1. The two new immunoadhesins were produced in 293F cells and affinity purified. Both recombinant proteins bound SIV gp130 in standard ELISA assays (not shown). All constructs were tested for their ability to neutralize SIV *in vitro*. The assays were performed by Monogram Biosciences, as described,[38] using several strains of SIV (Table 1). Rhesus albumin was used as a negative control.

The 4L6 immunoadhesin had potent activity against the SIVmac316 strain. The 4L6-RhAlb immunoadhesin performed similarly to 4L6, which indicates that there are no structural constraints for 4L6 when fused to albumin. The CD4(N4) immunoadhesin neutralized all SIV strains as expected. The CD4(N4)-RhAlb protein neutralized all strains except SIVmac316. This finding indicates that when the CD4 domain is linked to albumin, there are structural restraints that limit the effectiveness of the CD4 domain with SIVmac316. The RhAlb did not neutralize SIV, which was expected for the negative control.

Each of the four immunoadhesins—4L6, 4L6-RhAlb, CD4(N4), and CD4(N4)-RhAlb—was used to prepare rAAV-based delivery vectors and injected in rhesus macaques, 5 animals per group. The control group received either an empty vector (3 animals) or vector containing respiratory syncytial virus (RSV)-specific, single-chain antibody fused to rhesus albumin (2 animals). No toxicity associated with rAAV/immunoadhesin treatment was observed in treated animals based on phenotypic and biochemical evaluations. 8 weeks after rAAV injection and before SIV challenge, serum samples from all animals were analyzed for SIV neutralization activity (Table 2).

Table 1. Neutralization of SIV Strains by *In Vitro*-Produced Immunoadhesins

	SIVmac316	SIVmac251	SIVmac239	SIVE660	JRCSF	NL43
4L6	0.004	>50	>50	2.73	>50	>50
4L6-RhAlb	<0.001	>50	>50	19.61	>50	>50
CD4(N4)	0.035	0.38	2.35	0.01	3.18	0.03
CD4(N4)-RhAlb	>50	2.84	10.31	0.02	7.29	0.11
RhAlb	>50	>50	>50	>50	>50	>50

Recombinant proteins were expressed and purified, as described in Materials and Methods. Purified proteins were tested for *in vitro* neutralization activity by Monogram Biosciences (San Francisco, CA, USA) using a SEAP assay, as described in Materials and Methods. Immunoadhesin concentrations (in micrograms per milliliters), showing 50% neutralization activity against six SIV strains, are shown.

Consistent with the *in vitro* neutralization assay (see Table 1), the antibody-based immunoadhesins (4L6 and 4L6-RhAlb groups) had greater neutralizing activity against SIVmac316 than the CD4-based constructs. The CD4-based immunoadhesin (CD4(N4) group) showed broader neutralizing activity against several SIV strains. Individual animals from the 4L6-RhAlb group had higher variability in neutralizing serum titers than the animals from the 4L6 group. The animals from the group, which received the CD4(N4)-RhAlb construct, had the lowest SIV neutralizing titers in their sera. Similar to the *in vitro* results, in the serum of the CD4(N4)-RhAlb-immunized animals, the highest neutralizing activity was detected against SIVE660; however, no neutralizing activity was detected against SIVmac316.

All animals were challenged intravenously (i.v.) with a low dose (40 macaque infectious doses [mID]) of SIVmac316. Initially, 24 animals (all but one control, 14C255, which was challenged at a later time point, as indicated in Figure 2) were challenged at 8 weeks postvaccination. After a 20-week observation period, selected animals, including the 14C255 control animal, were challenged with a second and a third dose of SIVmac316. The results of these challenges are presented in Supplemental Information (see Figure S1 for the complete experimental design, and Figures S2 and S3 for SIV viral loads and immunoadhesin concentrations during the entire duration of the experiment). All animals from the control group and 3 out of 5 animals from the CD4(N4)-RhAlb group became infected as a result of the challenge, whereas all 15 animals from the 4L6, 4L6-RhAlb, and CD4(N4) groups (5 per group) remained virus free.

Immunoadhesin administration protected animals significantly in the 4L6 (Fisher's exact test, $p = 0.0079$), 4L6-RhAlb (Fisher's exact test, $p = 0.0079$), and CD4(N4) (Fisher's exact test, $p = 0.0079$) groups from infection, whereas it did not have a significant protective effect in the CD4(N4)-RhAlb group (Fisher's exact test, $p = 0.4444$) when compared to animals in the control group.

SIV viral loads for all animals are shown on Figure 2. Animals infected with SIV during the first challenge were not subjects for the additional challenges and remained under observation for about 2 years. We observed a different magnitude of SIV infection progression during the observation period. Two infected animals, one from the control group (14C263) and one from the CD4(N4)-RhAlb group (14C199), regained viral control within 16–28 weeks after infection and maintained low-to-undetectable viral load during the observation period. Three animals, two from the control group (14C205 and 14C202) and one from the CD4(N4)-RhAlb group (14C124) were sacrificed, due to SIV progression, which occurred between 16 and 80 weeks after SIV challenge (Figure 2). The remaining SIV-infected animals remained viremic during the observation period yet maintained CD4 T cell counts above 350 cells per microliter (Table S1).

Three animals, 2 that controlled the virus (14C199 and 14C263) and one animal that showed no evidence of infection (14C001), were treated with anti-CD8 antibodies on week 83 after the 1st SIV challenge (week 91 after AAV treatment), as described in Materials and Methods. Treatment resulted in near-complete depletion of CD8 T cells at 2–4 weeks after treatment (see Figure S4), followed by recovery. In both infected animals, CD8 T cell depletion correlated with a spike in viral load, reaching its maximum 2 weeks after anti-CD8 antibody injection (Figure 2).

Plasma viral load in the animals without evidence of SIV infection (14C001) remained undetectable after anti-CD8 treatment (not shown). Flow cytometry analysis of CD4/CD8 count and cell-associated markers of inflammation demonstrated a CD4 decline and changes in cell populations associated with SIV infection. There was no association between the immunoadhesin groups and changes in lymphocyte population frequency (Table S1 and Figure S5).

Serum levels of immunoadhesins were assessed using a SIVgp130-binding ELISA for Fc-based constructs (groups CD4(N4) and 4L6) and for albumin-containing constructs (groups CD4(N4)-RhAlb and 4L6-RhAlb), as described in Materials and Methods and Johnson et al.[24] The concentration of all 4 constructs reached maximum levels at 4–8 weeks after immunization with rAAV-based vectors (Figure 3). The immunoadhesin concentration in the 4L6 group was approximately 10-fold higher than in the CD4(N4) group, consistent with previous observations.[24] On the day of the SIV challenge, 8 weeks after the rAAV immunization, serum immunoadhesin concentrations were the following (in micrograms per milliliter ± SD): group 4L6, 20.2 ± 12.4; group CD4(N4), 5.8 ± 3.5; group 4L6-RhAlb, 22.0 ± 23.6; and group CD4(N4)-RhAlb, 2.7 ± 3.9. Three animals from

Table 2. Serum Neutralizing Activity against SIV Strains in Rhesus Macaques Treated with Immunoadhesins Delivered by an rAAV Vector

		SIVmac316	SIVmac251	SIVmac239	SIVE660	JRCSF	NL43	aMLV
Control	14C202	51	39	46	29	60	67	54
	14C205	61	60	43	38	63	89	65
4L6	14C001	1,726[a]	36	23	12	32	52	30
	14C070	791[a]	27	15	21	29	45	26
	14C206	2,239[a]	20	<10	10	20	32	19
	14C266	1,971[a]	50	33	33	39	63	43
	14C298	413[a]	35	22	19	28	39	27
4L6-RhAlb	14C078	976[a]	37	24	24	35	54	37
	14C081	35	26	16	23	39	49	31
	14C232	123[a]	36	21	18	32	51	35
	14C264	2,218[a]	52	39	35	53	87	49
	14C310	1,971[a]	38	22	20	32	48	24
CD4(N4)	14C085	331[a]	96	27	1,177[a]	34	415[a]	23
	14C098	445[a]	116[a]	49	1,698[a]	60	745[a]	44
	14C111	224[a]	141[a]	57	884[a]	69	288[a]	47
	14C123	257[a]	49	24	1,067[a]	38	292[a]	23
	14C160	204[a]	154[a]	29	763[a]	36	326[a]	27
CD4(N4)-RhAlb	14C099	50	62	23	756[a]	35	235[a]	30
	14C124	23	25	16	60	29	44	25
	14C134	19	32	<10	127[a]	28	60	23
	14C176	30	40	22	218[a]	41	96	23
	14C199	21	31	15	24	32	41	27
Z23	N/A	<100	<100	<100	<100	199[a]	2,481[a]	<100
Z23	N/A	<100	<100	<100	103	184[a]	1,990[a]	<100
Z23	N/A	<100	<100	<100	101	176[a]	1,713[a]	<100
Z23	N/A	<100	<100	<100	not done	175[a]	1,820[a]	<100

Animal serum samples collected 8 weeks after rAAV injection and before SIV challenge were tested for *in vitro* neutralization activity by Monogram Biosciences (San Francisco, CA, USA) using a SEAP assay, as described in Materials and Methods. Two animals from the control group, 14C202 and 14C205, were tested in the neutralization assay. Four positive samples (Z23) were also included by Monogram Biosciences as part of its established protocol. The Z23 antibody control is composed of broadly neutralizing HIV+ plasma and is present on all of the assay plates. The half-maximal inhibitory concentration (IC_{50}) values and curve shapes, resulting from testing the positive control plasma with the control viruses, confirm that the dilution series for each set of sera is correct and that the IC_{50}s of the controls fall within the assay acceptance range of 2.5-fold, 95% of the time. Titers shown are reciprocal serum dilutions representing 50% neutralization. N/A, not applicable.

[a]Titers considered to be highly neutralizing.

group CD4(N4)-RhAlb that became infected after the SIV challenge had the lowest neutralization titers and lowest plasma immunoadhesin concentration for this group. In 3 out of the 5 animals in the 4L6-RhAlb group, we observed a sharp drop in immunoadhesin concentration, soon after reaching peak level at weeks 4–6, although all 5 animals from this group were protected from the SIV challenge.

Immunoadhesin-specific immune responses were examined in animals who received rAAV immunization (Figure 4). The highest reactivity was observed against the transgene in animals from the CD4(N4)-RhAlb group. Two out of the 3 animals with the highest reactivity (14C199 and 14C134) were infected during the SIV challenge. High levels of immunoadhesin-specific antibodies were also detected in 2 out of the 5 animals in the 4L6-RhAlb group. In the 4L6 and the CD4(N4) groups, detectable levels of immunoadhesin-specific antibodies were relatively low and in most animals, had the tendency to decline after 12–20 weeks past rAAV immunization. At week 8, the only difference between CD4(N4) and CD4(N4)-RhAlb groups was significant ($p < 0.01$ by 2-way ANOVA with post hoc Tukey's test). We did not see any effect of sex on SIV infection (Fisher's exact test, $p > 0.05$) or on immunoadhesin expression or levels of anti-immunoadhesin antibodies (Mann-Whitney test, $p > 0.05$).

DISCUSSION

In this study, we extended our previous work by Johnson et al.[24] of 4L6 and CD4(N4) immunoadhesins. Whereas we used the same 4L6 construct as reported previously, a single amino acid change was introduced in the CD4-based immunoadhesin, replacing

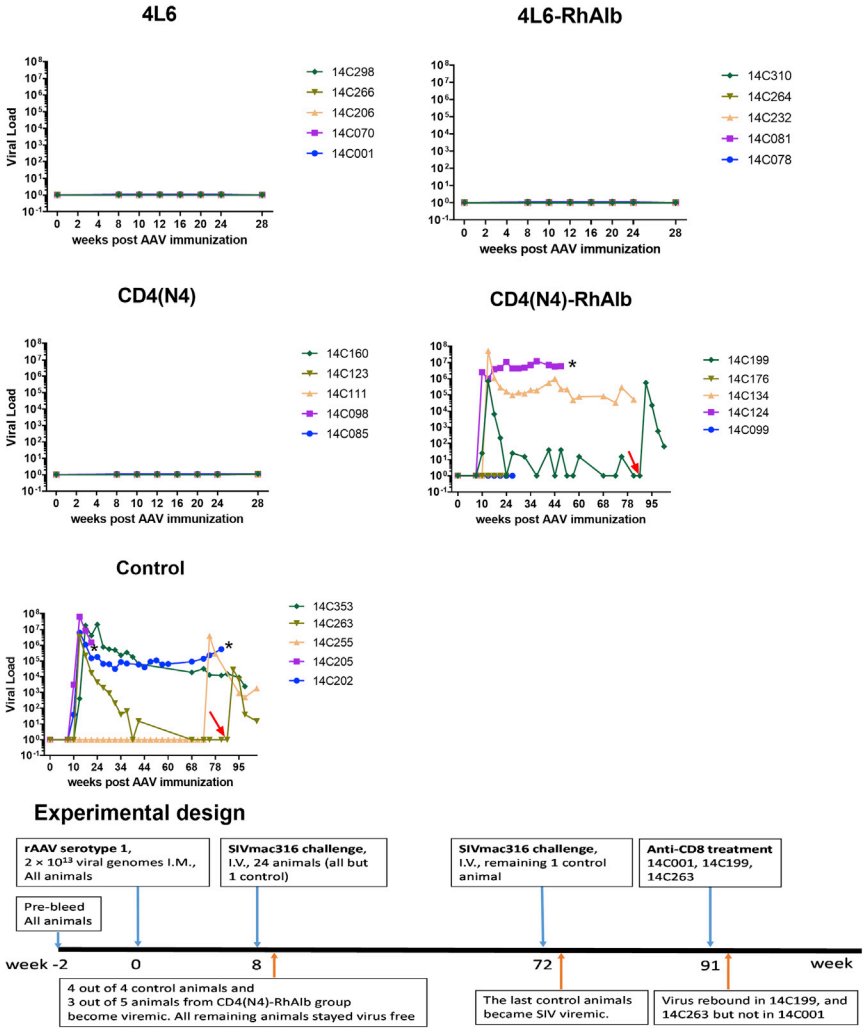

Figure 2. Detection of SIV in Plasma after Challenge

All animals received a low-dose SIV challenge (all animals but 14C255, which was challenged with the same dose of SIV but at later time point; for details see Supplemental Information) at week 8 after AAV immunization. Three animals (14C205, 14C202, and 14C124) were sacrificed due to SIV progression, indicated with an asterisk (*). Three animals were treated with anti-CD8 antibodies on week 83 after SIV challenge, 2 animals that controlled the virus (14C199 and 14C263; indicated with red arrow) and one animal that showed no evidence of infection (14C001). Plasma viral loads were determined using a real-time reverse transcriptase PCR by Leidos Biomedical Research, NCI (Frederick, MD, USA), as described in Materials and Methods. Bottom of figure shows brief study design.

isoleucine with asparagine (N) at the position 39, which has been reported to increase the SIV neutralization potency.[20,39]

As predicted from our previous study,[24] the 4L6 immunoadhesin was highly efficient in neutralizing the SIVmac316 strain *in vitro* but not other SIV strains. This immunoadhesin construct is based on an antibody isolated from a rhesus macaque that was infected with SIVmac316 and is very potent in neutralizing SIVmac316 *in vitro*. The 4L6-RhAlb construct neutralized SIVmac316 at an even lower concentration than 4L6, suggesting that albumin fusion does not affect the antibody-binding activity. CD4-based immunoadhesins were not as potent against SIVmac316 as the antibody-based immunoadhesins, which is consistent with previous observations.[24] However the CD4(N4) immunoadhesin neutralized all SIV strains tested, confirming the hypothesis that CD4-based immunoadhesins provide a wide spectrum of neutralization against SIV strains. The CD4(N4)-RhAlb immunoadhesin neutralized all strains except SIVmac316. This finding was unexpected, since CD4(N4) and CD4(N4)-RhAlb immunoadhesins share the same CD4 domain. It is possible, however, that when the CD4 domain is linked to albumin, it introduces structural restraints that affect the CD4 binding. Although CD4(N4)-RhAlb neutralization activity against other SIV strains was reduced as compared to Fc-based fusion (CD4(N4)), only SIVmac316 neutralization was significantly affected.

Whereas the current study is building on results from our previous study,[24] there are some relevant differences. In our initial study, 3 animals per group were used for the SIV challenge experiment,[24] whereas in the current study, we had 5 animals per group. In both studies, we observed 100% protection with the 4L6 construct (3 out of 3 animals in the early study[24] and 5 out of 5 animals in the current study) against SIV challenge. In this study, we used the CD4 domain with a single amino acid change replacing isoleucine with asparagine at the position 39. In the previous study, 2 out of the 3 animals were protected in the CD4(N4) group,[24] whereas in the current study, we observed protection in 100% (5 out of 5) of the animals in the CD4(N4) group. Our results with the CD4(N4) immunoadhesin are also consistent with the recently published study employing CD4-immunoglobulin (Ig) fusion for SIV immunoprophylaxis.[14] In both studies, 100% protection was achieved after the initial challenge dose, although there are substantial differences in the construct design and the challenge details between the two studies.

Low neutralization activity in the sera from the CD4(N4)-RhAlb group correlated with partial protection in *in vivo* tests from the same group. Two protected animals (14C099 and 14C176) had the highest neutralization titers against SIV316 within the CD4(N4)-RhAlb group, although not reaching reference level (Table 2). Two out of the 5 animals in the 4L6-RhAlb group (14C081 and 14C232) did not have detectable immunoadhesins in their plasma at the time of the SIV challenge at week 8 (Figure 3) and had low to no

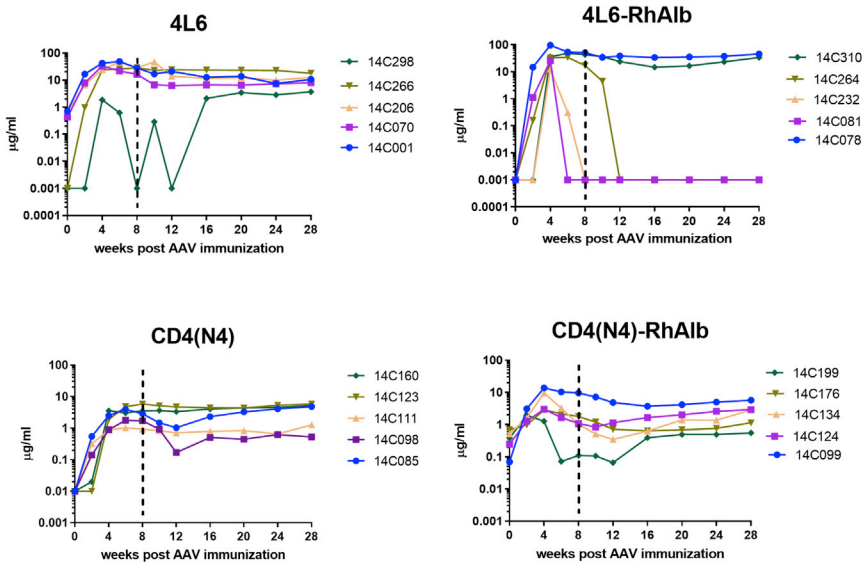

Figure 3. Serum Concentration of Immunoadhesins and Anti-SIV Antibodies after Gene Transfer and SIV Challenge

Immunoadhesin concentrations in serum of animals immunized with rAAV-based vectors at week 0 and challenged with SIV on week 8 (indicated with dotted lines) were determined using ELISA, as described in Materials and Methods. Immunoadhesin concentrations are expressed in micrograms per milliliter.

detectable neutralization titers (Table 2). Those animals, however, remained protected against SIV challenge. These data are consistent with the report that very low serum concentration of an antibody-like molecule, in this case, a CD4-IgG2 construct, may be sufficient for macaque protection.[40]

Anti-CD8 treatment resulted in transient viral rebound in the 2 animals reaching peak viremia between 28,000 and 1,400,000 copies per milliliter, followed by decline. None of the animals regained complete viral control after anti-CD8 treatment during the observation period (Figure 2). We do not have evidence that immunoadhesin expression contributed to viral control postinfection, since one of the animals controlling the virus was from the untreated control group. Animals showing viral control had evidence of "protective" TRIM5-TFP/Cyp and 1 Mamu-B*017 genotypes. However, several animals with the TRIM5-TFP/Cyp genotype did not control the infection (see Table S2). We also treated one animal with anti-CD8 antibodies (14C001), and this animal did not show evidence of infection after challenge. As expected, anti-CD8 treatment did not result in the emergence of detectable virus, suggesting that the protected animals do not have an SIV viral reservoir.

The host immune response has been described as a limiting factor in several studies of antibodies and antibody mimetic molecules.[14–19] Host response varies significantly based on construct design and from one animal to another.[14,16–18] We detected the highest levels of anti-immunoadhesin antibodies in animals expressing the CD4(N4)-RhAlb construct. This group of animals had the lowest level of protection against the first low dose SIV challenge. The immunogenicity of CD4(N4)-RhAlb in rhesus macaques may be partially responsible for poor animal protection. Although several animals in both albumin-conjugated immunoadhesin groups had high levels of anti-transgene antibodies, the overall difference in their anti-immunoadhesin immune response between albumin and Fc-conjugated groups did not reach statistical significance ($p > 0.05$ by Mann-Whitney test). Although albumin is not inherently immunogenic,[29] fusion with other peptides may affect the immunogenicity of the resulting molecules. There needs to be improvements on the design of albumin fusion constructs that will reduce immunogenicity and will retain neutralization activity in order to consider this approach for further *in vivo* testing. A recent publication demonstrating that IgG2-Fc-based constructs induce a reduced host response[14] is consistent with our observations of lower host response against IgG2-Fc-based 4L6 and CD4(N4) immunoadhesins. Anti-transgene immune responses will likely play a critical role in any future application of immunoadhesin-like molecules in humans. In a phase I human application of native broadly neutralizing antibodies by the rAAV vector, anti-transgene antibodies were detected in the majority of individuals in the intervention group.[25]

In conclusion, our approach shows promise as a potential HIV prophylactic strategy by delivering and maintaining long-lasting expression of protective molecules. In addition, the general strategy of "immunoprophylaxis by gene transfer" can be applied to other difficult vaccine targets, including hepatitis C virus, malaria, RSV, and tuberculosis.

MATERIALS AND METHODS

Anti-SIV Immunoadhesin Gene Constructs

The detailed design and preparation of the 4L6 and CD4(N4) immunoadhesins have been described previously.[24] Briefly, DNA was synthesized (Geneart) using optimized codons. For the 4L6 construct, the variable domain of SIV gp130-specific Fab was joined by a 15-amino acid glycine-serine $(G_4S)_3$ linker, and a synthetic signal peptide for optimized secretion 56 was placed at the 5′ end. The Fc fragment was rhesus IgG2 cloned from lymphocyte RNA. CD4-based constructs contain the rhesus CD4 signal sequence, followed by the D1/D2 domains of rhesus CD4. The CD4 domain contains a single amino acid change introducing an N at the position 39 (I39N-CD4(N4)), which increases the SIV neutralization potency.[20,39] Each construct was placed between a cytomegalovirus (CMV) promoter[41] and a synthetic polyadenylation signal.[42] For a schematic representation of constructs, please see Figure 1.

The CD4-albumin-CD4 construct is identified as "CD4(N4)-RhAlb" and contains rhesus albumin with CD4(N4) fused to both the N and C terminus (N-C). The single-chain variable fragment

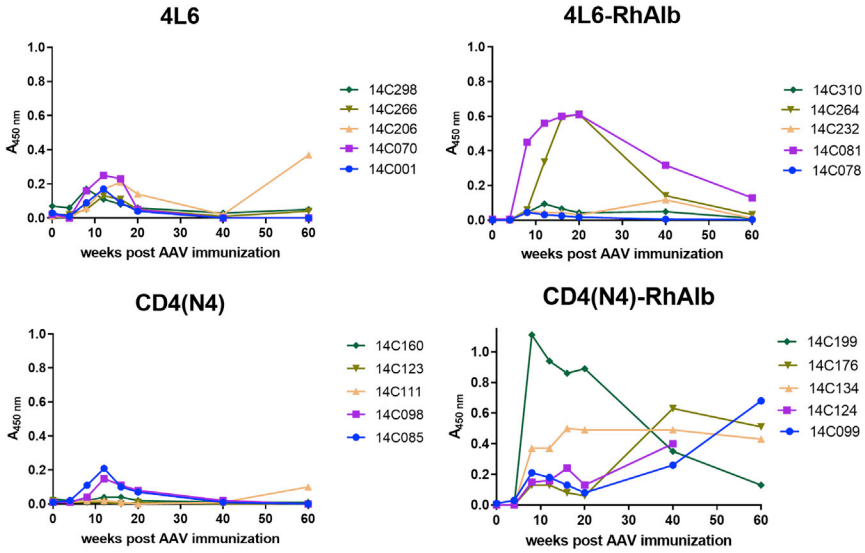

Figure 4. Anti-Immunoadhesin Antibody Titers in Immunized Animals

Concentrations of anti-immunoadhesin antibodies in serum (1:100 dilution) were determined using ELISA, as described in Materials and Methods, using plates coated with purified immunoadhesins corresponding to each group as indicated. The results are expressed as ELISA optical density absorbance at 450 nm (OD A450 nm).

(scFv)-albumin-scFv construct (4L6- RhAlb) consists of the scFv domain of the rhesus antibody "4L6" that has been previously described;[24] all components are fused in N-C orientation. A similar scFv-albumin-scFv construct was made with a single-chain antibody of irrelevant specificity (against RSV) and was used to prepare the negative control rAAV-based vector.

Recombinant Proteins

HeLa cells were transfected (Superfect; QIAGEN) with plasmids containing the gene constructs, and proteins were purified from the medium using protein-A (Nunc International). Purified proteins were quantified by ELISA using purified rhesus IgG as a standard (Bethyl Laboratories).

Adeno-Associated Viral Vectors

AAV serotype 1 vectors were produced and purified as previously described.[43–45] Titers ranged between 2×10^{12} and 1×10^{13} vector genomes per milliliter.

Rhesus Macaques Immunization and Treatment

Animals

Twenty-five rhesus macaques of Indian origin, 40% female, evenly spread among 5 groups, 31–40 months old at the start of the experiment, were purchased from Covance (Alice, TX, USA) and housed in the vivarium at the Children's Hospital of Philadelphia Research Institute, in accordance with standards set forth by the Association for Assessment and Accreditation of Laboratory Animal Care (protocol #16-001237). For animal genotypes, see Table S2. All animals were negative for antibodies to SIV, simian type D retrovirus, and simian T cell lymphotropic virus type 1. The body weights at the time of immunization ranged from 2.7 to 4.2 kg. Each immunized animal received 2×10^{13} vector genomes divided into 4 equal portions (0.75 mL each), delivered by 4 separate (2 in each quadriceps) deep intramuscular injections. The animals were divided into 5 groups (40% female in each group). Each study group received an rAAV vector containing one of four immunoadhesins. Animals from the control group received either an empty vector (3 animals) or a vector containing an RSV-specific, single-chain antibody fused to rhesus albumin (RSV-Fc-RhAlb; 2 animals).

CD8 Depletion Studies

For CD8 depletion studies, animals received a single intravenous infusion of 50 mg/kg of the anti-CD8α mouse/rhesus complementarity-determining region (CDR)-grafted rhesus IgG1 antibody MT807R1 (Nonhuman Primate Reagent Resource; Mass-Biologics, Mattapan, MA, USA). CD8 T cell counts and viral loads were assessed biweekly following anti-CD8 infusion.

SIV Neutralization Assay

Purified proteins or macaque sera were tested for *in vitro* neutralization activity by Monogram Biosciences (San Francisco, CA, USA), using a secreted, engineered alkaline phosphatase (SEAP) reporter cell assay, described by Means et al.[38]

Immunoadhesin Concentrations

Immunoadhesin concentrations were measured as described.[24] For detection of the Fc-based immunoadhesins (4L6 and CD4(N4)), goat anti-human IgG-Fc horseradish peroxidase (HRP)-conjugated secondary antibody (Bethyl Laboratories; A80-104P) was used. For detection of albumin-based immunoadhesins (CD4(N4) RhAlb and 4L6-RhAlb), goat anti-human albumin HRP-conjugated, cross-adsorbed secondary antibody (Bethyl Laboratories; A80-229P) was used. Quantification was achieved by extrapolation from the standard curve using software from Molecular Devices (coefficient of linearity, ≥ 0.99).

Anti-Transgene Antibody Responses

Anti-immunoadhesin antibody responses were measured as described.[24] Briefly, plates were coated with purified immunoadhesins. An IgG1 isotype-specific secondary antibody was chosen to avoid cross-reactivity with the IgG2 immunoadhesins.

SIV Challenge

Infectious SIVmac316 stock was generated and assayed as described.[24] The stock used in this study contained 357 ng/mL of p27. Animals were infected intravenously with 1 mL of the diluted stock, which was estimated to contain approximately 40 mID or 143 pg of SIV p27 per animal. Viral loads in plasma were determined

using a quantitative real-time reverse transcriptase PCR assay, as previously described,[46] by qPCR (Leidos Biomedical Research, National Cancer Institute [NCI], Frederick, MD, USA), with a limit of detection of log 1.2 (or 15 copies/mL). Selected animals were challenged with additional doses of SIV. The results of the second and third challenges are presented in Supplemental Information.

Flow Cytometry

Flow cytometry was performed on an Accuri C6 and LSRII analyzers (BD Biosciences). In order to determine CD4/CD8 T cell count, the following antibody panel was used: anti-CD45 fluorescein isothiocyanate (FITC) conjugated (BD Biosciences; cat #557803); anti-CD3 allophycocyanin (APC) conjugated (BD Biosciences; cat. #557597); anti-CD4 phycoerythrin (PE)-cyanine-7 (Cy7) conjugated (BioLegend; cat. #317414); anti-CD8 PE conjugated (BD Biosciences; cat. #555367) on an Accuri C6 analyzer.

Monocyte and B cell activation markers were evaluated using the following panel: anti-CD14 PE conjugated (BD Biosciences; cat. #555398); anti-CD16 Brilliant Violet (BV)605 conjugated (BD Biosciences; cat. #563172); anti-CD163 Alexa Fluor 647 conjugated (BD Biosciences; cat. #562669); anti-CD20 APC-Cy7 conjugated (BD Biosciences; cat. #335794); anti-CD21 PE-Cy7 conjugated (BD Biosciences; cat. #561374); anti-CD27 FITC conjugated (BD Biosciences; cat. #555440).

T and natural killer (NK) cell activation markers were evaluated using the following panel: anti-CD3 peridinin-chlorophyll protein (PerCP) conjugated (BD Biosciences; cat. #552851); anti-CD4 PE-Cy7 conjugated (BD Biosciences; cat. #560644); anti-CD8 APC-Cy7 conjugated (BD Biosciences; cat. #557760); anti-programmed death 1 (PD-1) APC conjugated (BD Biosciences; cat. #329908); anti-CD38 FITC conjugated (Novus; cat. #NBP2-47908F); anti-CD45RA BV421 conjugated (BD Biosciences; cat. #740083); anti-CD62 ligand (CD62L) PE conjugated (BD Biosciences; cat. #341012); anti-CD16 BV605 conjugated (BioLegend; cat. #302046).

Viability staining was done using blue-fluorescent reactive dye (Life Technologies; cat. #L23105).

Statistical Analyses

Fisher's exact probability test (two tailed), 2-way ANOVA with post hoc Tukey's test, and Mann-Whitney test were used to examine the difference between groups, as indicated with GraphPad Prism 7.04.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.04.019.

AUTHOR CONTRIBUTIONS

S.S., B.C.S., M.J.C., T.L., C.M.D., V.P., R.T., and A.K. optimized protocols and conducted the experiments. S.S. and V.P. performed statistical analysis. S.S., B.C.S., V.P., P.R.J., and S.D.D. drafted and revised the manuscript. P.R.J. and S.D.D. provided study oversight and obtained funding for the project.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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