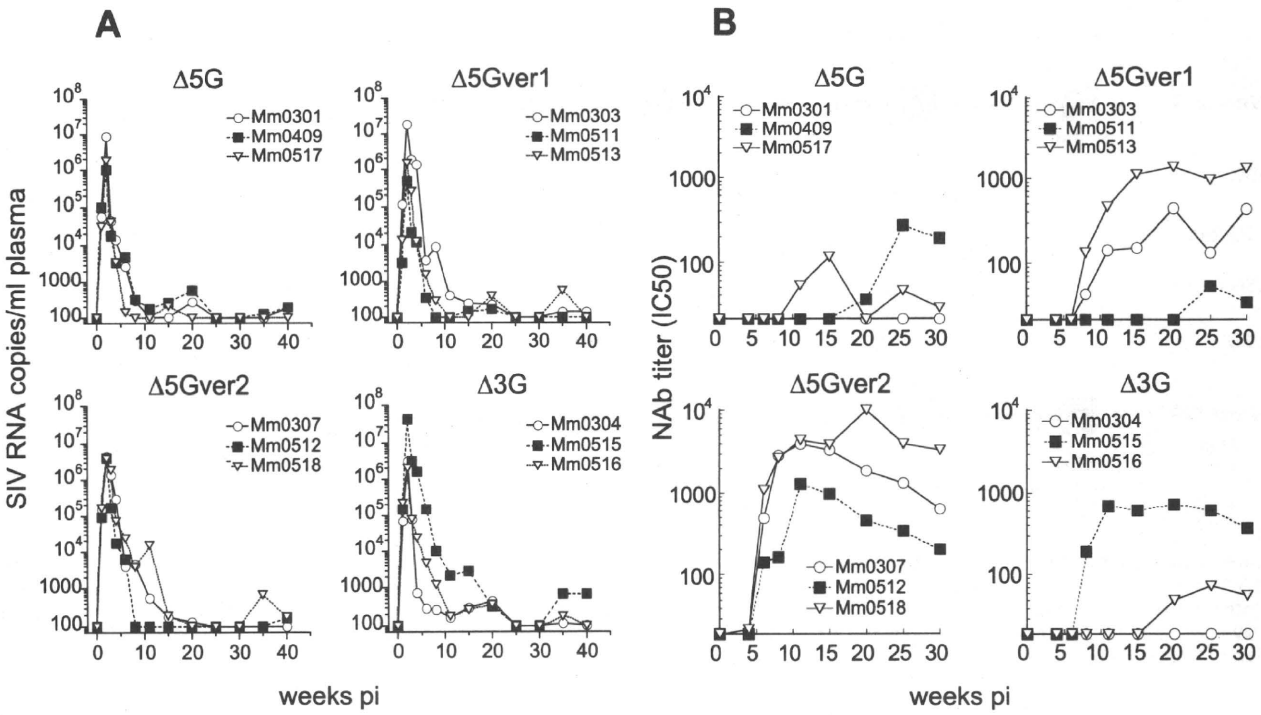

Figure 2. Viral loads and neutralizing antibodies in macaques infected with each of 4 deglycosylated SIV mutants. Twelve animals were divided into 4 groups consist of 3 animals each and infected with each of 4 deglycosylation mutants (Δ5G, Δ5Gver-1, Δ5Gver-2, and Δ3G). (A) Plasma viral loads were determined by real-time RT-PCR with SIVmac239 primers and probe set. (B) NAb responses against each respective infecting virus were measured in CEMx174/SIVLTR-SEAP system. NAb titers were indicated as the reciprocal of the dilutions of the plasma from the vaccinees yielding 50% inhibition (IC_{50}).
doi:10.1371/journal.pone.0011678.g002

primary infection with similar kinetics (Fig. 2 A) suggesting that NAb were most likely not a critical factor for containment of the acute infection in these animals.

We previously found that animals vaccinated with Δ5G completely resist infection when challenged with the parental pathogenic SIVmac239 [3], showing minimal if any replication of the challenge virus for more than 10 years. A similar homologous challenge was performed in a subset of animals that received the deglycosylation mutants in the present study. Thus, one of the three "immunized" animals from each group was challenged with a high dose (1000 $TCID_{50}$) SIVmac239 at 40 weeks following "vaccination" and plasma viral loads were determined (Fig. 3). As previously reported with Δ5G, a near-sterile immunity against challenge with SIVmac239 was not only noted with the Δ5G but also seen with our other three new deglycosylated SIV mutants, Δ5G-ver1, Δ5G-ver2 and Δ3G (Fig. 3). These results indicate that all 3 new vaccine versions possess similar equally high protective potential against the homologous, wild type SIVmac239 as the original Δ5G.

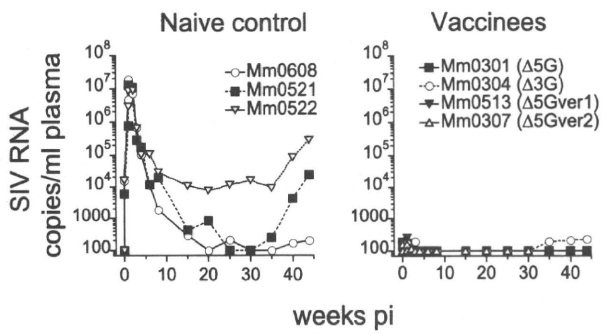

Figure 3. Plasma viral RNA loads in the homologous SIVmac239 challenge. Three naïve rhesus macaques (Mm0608, Mm0521, Mm0522) and 4 vaccinees (Mm0301, Mm0304, Mm0513, Mm0307), i.e. one animal from 4 deglycosylated SIV infection groups, were challenged intravenously with 1000 $TCID_{50}$ of SIVmac239. Plasma viral loads were determined by real-time RT-PCR with SIVmac239 primers and probe set.
doi:10.1371/journal.pone.0011678.g003

Protection of the vaccinated macaques against heterologous challenge infection

The remaining eight animals (2 per group) vaccinated with each of the 4 vaccine versions (Δ5G, Δ5G-ver1, Δ5G-ver2 or Δ3G) and 3 of the four animals that were vaccinated (described in the above paragraph) and challenged with SIVmac239 (Mm0307 died of SIV unrelated causes) were challenged with a high dose (1000 $TCID_{50}$) of SIVsmE543-3 delivered intravenously. Additional three naïve animals served as a control for this heterologous challenge experiment (Fig. 4 A). VL were monitored until 80 weeks post challenge (pc) using real time RT-PCR primer pairs and probes that distinguished the detection of SIVmac239 and SIVsmE543-3.

The 3 naïve control macaques infected with SIVsmE543-3 exhibited a peak VL of ~10^7 copies/ml at 2 weeks pi which is essentially similar to those we have routinely noted following infection with SIVmac239 with a few exceptions. Notably, the set point VL in SIVsmE543-3 was more than 10^5 copies/ml in 2 animals which is at least 1-log higher than that noted in animals infected with SIVmac239 (Figs. 3 and 4 A). We reason that SIVsmE543-3 is likely to be more pathogenic than SIVmac239 for

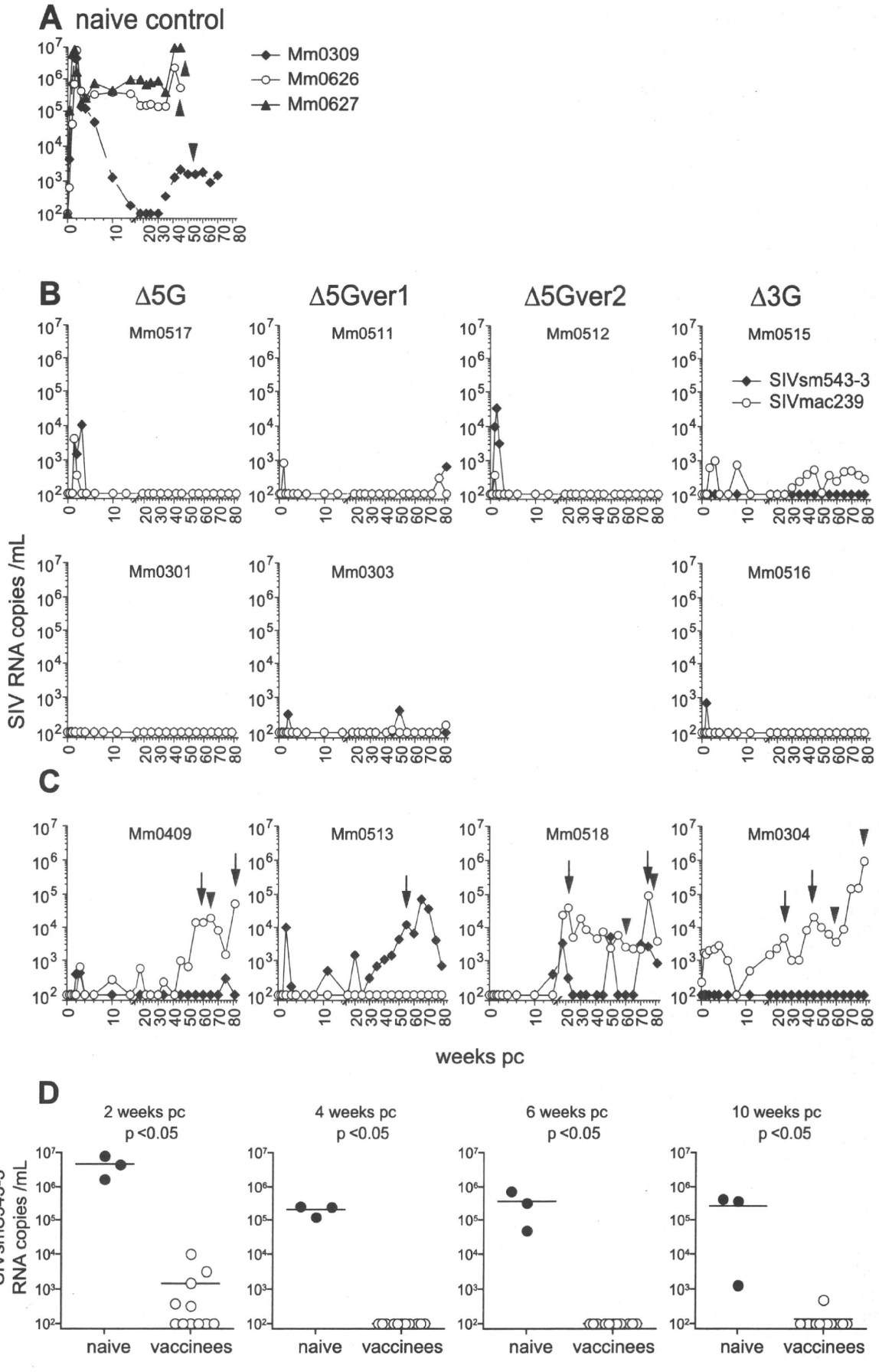
A naive control
Mm0309
Mm0626
Mm0627
B
Δ5G
Δ5Gver1
Δ5Gver2
Δ3G
Mm0517
Mm0511
Mm0512
Mm0515
SIVsm543-3
SIVmac239
Mm0301
Mm0303
Mm0516
SIV RNA copies /mL
C
Mm0409
Mm0513
Mm0518
Mm0304
weeks pc
D
2 weeks pc
p <0.05
4 weeks pc
p <0.05
6 weeks pc
p <0.05
10 weeks pc
p <0.05
SIVsmS543-3
RNA copies /mL
naive
vaccinees

Figure 4. Plasma viral RNA loads in the heterologous SIVsmE543-3 challenge. Three vaccine-naïve animals (Mm0309, Mm0626, Mm0627) (A) and 11 vaccinees (B, C) were challenged intravenously with 1000 $TCID_{50}$ of SIVsmE543-3. The vaccinees were divided into controllers (B) and non-controllers (C) based on control of vaccine and heterologous challenge infection. VL were determined by two sets of real-time RT-PCR for gag sequence of SIVsmE543-3 (closed diamonds) and SIVmac239 (open circles) respectively. For analysis of SIV sequence, PBMC and plasma were collected at the time-points indicated by arrows and arrowheads, respectively. (D) VL of the 11 vaccinees were statistically compared with those of 3 vaccine-naïve controls at 2, 4, 6 and 10 weeks pc. Significant difference between vaccinees and controls at each time points are shown (Mann-Whitney test).
doi:10.1371/journal.pone.0011678.g004

our Burmese rhesus macaques. Indeed, these 2 animals developed AIDS and were euthanized at 46 weeks pi, which is significantly faster than the time that we have noted for disease progression following SIVmac239-infection of these Burmese monkeys [3,8,15,16,17].

All 11 vaccinated animals contained the primary challenge with SIVsmE543-3 (Figs. 4A, B, and C). At 2 weeks pc, average VL for the vaccine groups were >3-log lower than those of naïve control animals (Fig. 4D). Thus, 5 of the 11 vaccinated animals (Mm0511, Mm0515, Mm0301, Mm0518, and Mm0304) contained the acute challenge below the level of detection (100 copies/ml) at all times, 3 animals (Mm0303, Mm0516, and Mm0409) showed marginal replication of SIVsm543-3 (329–700 copies/ml) during weeks 1–3 pc, while the other 3 animals (Mm0517, Mm0512, and Mm0513) showed viremia with $1–3\times10^4$ copies/ml during weeks 1–3 pc but these replication peaks were transient and by 4 weeks pc, all vaccinated animals controlled SIVsmE543-3 to undetectable levels (Figs. 4B, C and D). Interestingly, transient replication of the vaccine viruses was also detected in 6 animals after the SIVsmE543-3 challenge (Mm0517, Mm0511, Mm0512, Mm0515, Mm0409, and Mm0304) (Figs. 4 B and C), suggesting reactivation of the deglycoylated virus upon super-infection with SIVsmE543-3. Statistical analysis of these data led us to conclude that the vaccinated animals significantly controlled SIVsmE543-3 replication at least during the acute phase up to 10 weeks pc irrespective of their MHC genotypes (Fig. 4 D). These results, taken together indicate that each of the deglycosylated vaccines utilized has the potential of inducing protective immunity against a potentially highly pathogenic heterologous challenge virus.

We next evaluated the containment of the challenge virus infection during the chronic-phase compared with the one observed after homologous challenge with SIVmac239 (Fig. 3 and ref [3]). Based on longitudinal VL (of either vaccine or challenge virus), the vaccinated animals were divided into two groups. One group of 7 animals, which we termed as "controllers", comprised animals which controlled the challenge virus almost completely for the 80 weeks of follow-up pc (Fig. 4 B). Detailed analyses of VL showed complete control of the SIVsmE543-3 challenge in two of the animals (Mm0301 and Mm0515) over time. Similar potent antiviral control (except for small VL peaks during the acute-phase) were noted in three of the animals (Mm0512, Mm0516 and Mm0517). The last two "controllers" showed only occasional VL blips during the chronic-phase (Mm0303 and Mm0511). However, challenge with SIVsmE543-3 induced persistent low vaccine VL in Mm0515, while the challenge virus remained undetectable.

In what we termed as the non-controller group of 4 animals, VL gradually increased with time (Fig. 4 C). The evolving replicating viruses were found to consist of the challenge virus in Mm0513, whereas they were apparently vaccine viruses in the remaining three (Mm0409, Mm0518 and Mm0304). In the latter three, it appeared as if vaccine viruses were reactivated upon challenge with heterologous virus. These three eventually developed AIDS and were euthanized, whereas Mm0513 has not shown any disease manifestation >80 weeks pc. These four animals were regarded as poor or non-controllers.

These results indicate that the deglycosylated, live attenuated SIV viruses function as effective vaccines and possess potential to induce near-sterile, long-lasting immunity against the heterologous virus in a significant, albeit not all vaccinated animals. Also these results demonstrated that all 4 deglycosylation mutants exhibited similar vaccine efficacy based on the ratio of controllers and non-controllers (Figs. 4 B and C).

Adaptive immune responses in vaccinees

In efforts to investigate immune correlates of protection against the heterologous challenge in vaccinees during acute and chronic infection, we examined adaptive immune responses against vaccine and challenge viruses. As described, the levels of NAb responses against vaccine virus varied among the vaccinees, partly due to the differences in N-glycosylation in gp120 (Figs. 1 and 2B). The differences in the NAb responses in vaccinees were maintained even after challenge with the heterologous virus. Whereas the Δ5G-ver2-vaccinated animals elicited the highest level of NAb, the Δ3G-vaccinated animals elicited the lowest level of NAb. In addition, the Δ5G and Δ5Gver1-vaccinated animals elicited intermediate NAb responses (Fig. 5). Regardless of these differences, all of the vaccinees successfully contained acute-phase VL, before diverging into controllers and non-controllers during the chronic infection-phase (Figs. 4 B and C). Thus, vaccine induced NAb responses did not correlate with protection from challenge virus infection during either acute or chronic infection. In addition, we could not detect any appreciable NAb against SIVsmE543-3 in any of the vaccinees throughout the observation period (Fig. 5). Although NAb was reasoned to exert immune pressure driving the emergence of mutants with altered N-glycosylation in HIV/SIV infections [18,19,20], no significant association was observed between NAb responses and the emergence of the mutants in non-controllers (Figs. 4 and 5).

We next examined cellular responses specific to the viral proteins in the PBMC utilizing the IFN-γ ELISPOT assay against pools of peptides spanning the entire proteins of both SIVsmE543-3 and SIVmac239. Specific T cell responses to SIVmac239-peptides paralleled those to SIVsmE543-3-peptides in all of vaccinees, and therefore vaccine-elicited SIV specific T cells were assumed to cross-react with SIVsmE543-3 infected cells (Fig. 6). However, no obvious quantitative correlation was found between the overall specific T cell responses and either good, poor or the lack of control of viremia throughout the observation period (Fig. 4). It is of interest to note that more than half of the SIV specific T cell responses appeared directed against epitopes localized within the SIV-Gag protein in most of the vaccinated animals (Fig. 6) which suggests that a potential association exists between gag specific T cell response with control of viremia. These findings are consistent with previous reports that suggest that the magnitude of Gag-specific T cell response correlates with control of HIV/SIV viremia in not only HIV-1-infected cohorts [11,21] but also in macaques included in vaccine studies [8,16].

Taken together, whereas these results indicate no appreciable correlation between NAb response and control of heterologous challenge intravenous infection, there may exist a potential role of virus specific cellular responses in the control of viral replication.

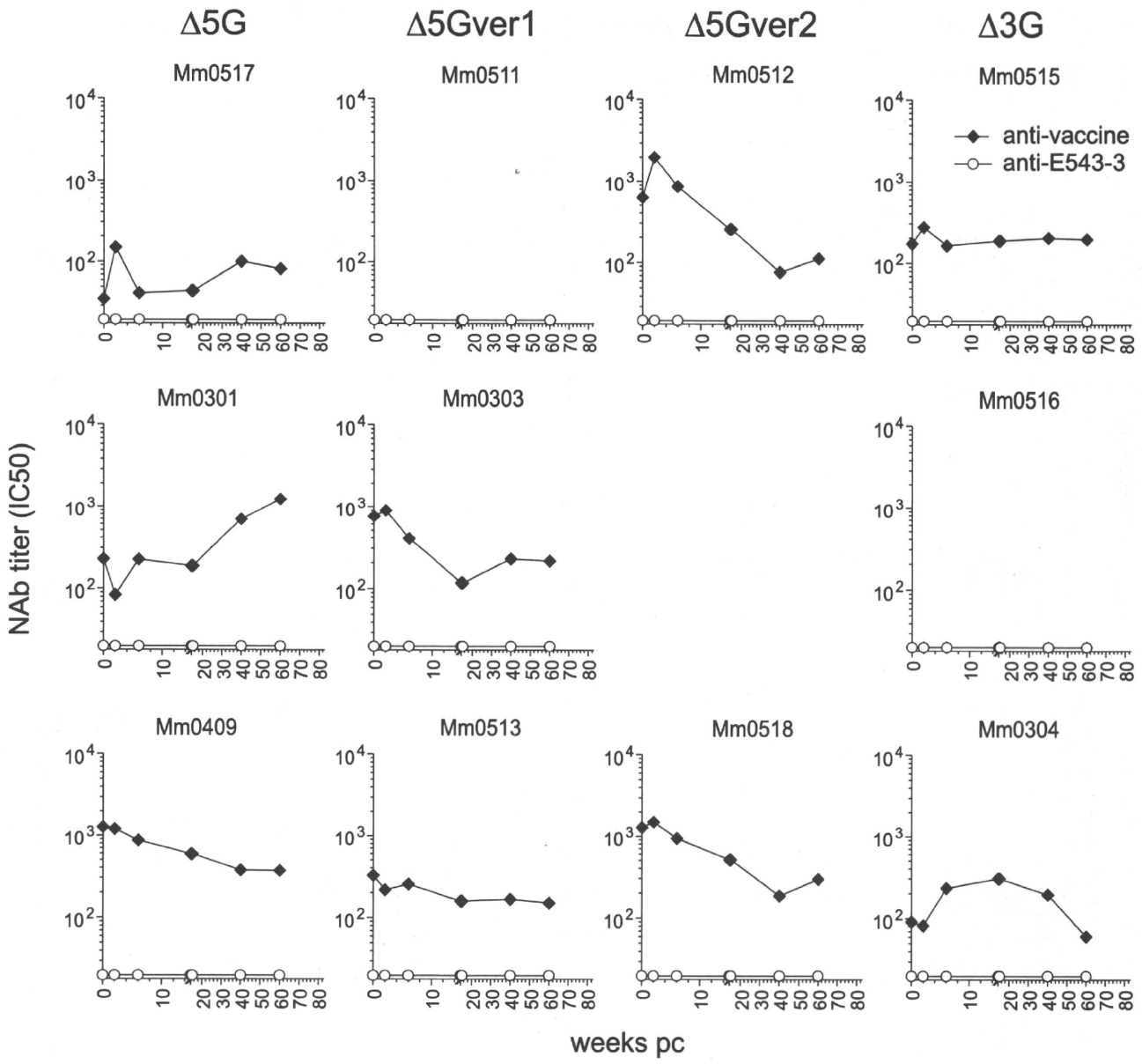

Figure 5. Neutralizing antibodies (NAb) response in the vaccine recipients. NAb responses against vaccine viruses (closed squares) and challenge virus (open circles) were measured in CEMx174/SIVLTR-SEAP system. NAb titers were indicated as the reciprocal of the dilutions of the plasma from the vaccinees yielding 50% inhibition (IC_{50}).
doi:10.1371/journal.pone.0011678.g005

Emergence of escape mutants with increased N-glycosylation sites in gp120 by recombination and single point mutations

In efforts to understand the mechanisms involved in the loss of control of viremia in the 4 non-controllers, we sequenced the emerging viruses using PBMC collected at time points indicated by arrows in Fig. 4 C. Sequence analysis of viruses isolated from Mm0513 confirmed that only the challenge virus with a 9 nucleotide deletion was replicating in this animal (Fig. 7). Whereas the vaccine virus was detected in the PBMC from Mm0304 collected at 25 weeks pc, a recombinant virus was predominantly present in the PBMC samples collected at 45 weeks pc from this animal (Fig. 7). Viruses with multiple recombinations were also found in Mm0409 and Mm0518 (Fig. 7). To examine if the recombination that we detected in the PBMC DNA was representative of the replicating viruses, we performed nested PCR utilizing primer pair sets aimed at the detection of putative recombination sites on RNA obtained from plasma from each animal (File S1). Consistent with the results obtained from PBMC DNA, the recombinants were also found in plasma RNA samples in all 3 animals, whereas only a few SIVsmE543-3 sequences were detected in Mm0518 (Table 2).

As noted above, while attenuated vaccine viruses have 18 or 20 N-glycosylation sites, the pathogenic strains, SIVmac239 and SIVsmE543-3 have 23 and 22 *N*-glycosylation sites, respectively (Fig. 1). We noticed that the gp120-encoding region of all replicating viruses in the chronic phase post challenge originated from the SIVsmE543-3 isolate regardless of recombination (Fig. 7). This resulted in restoration of N-glycosylation sites, the number of which was analyzed. We sequenced the PCR products amplified

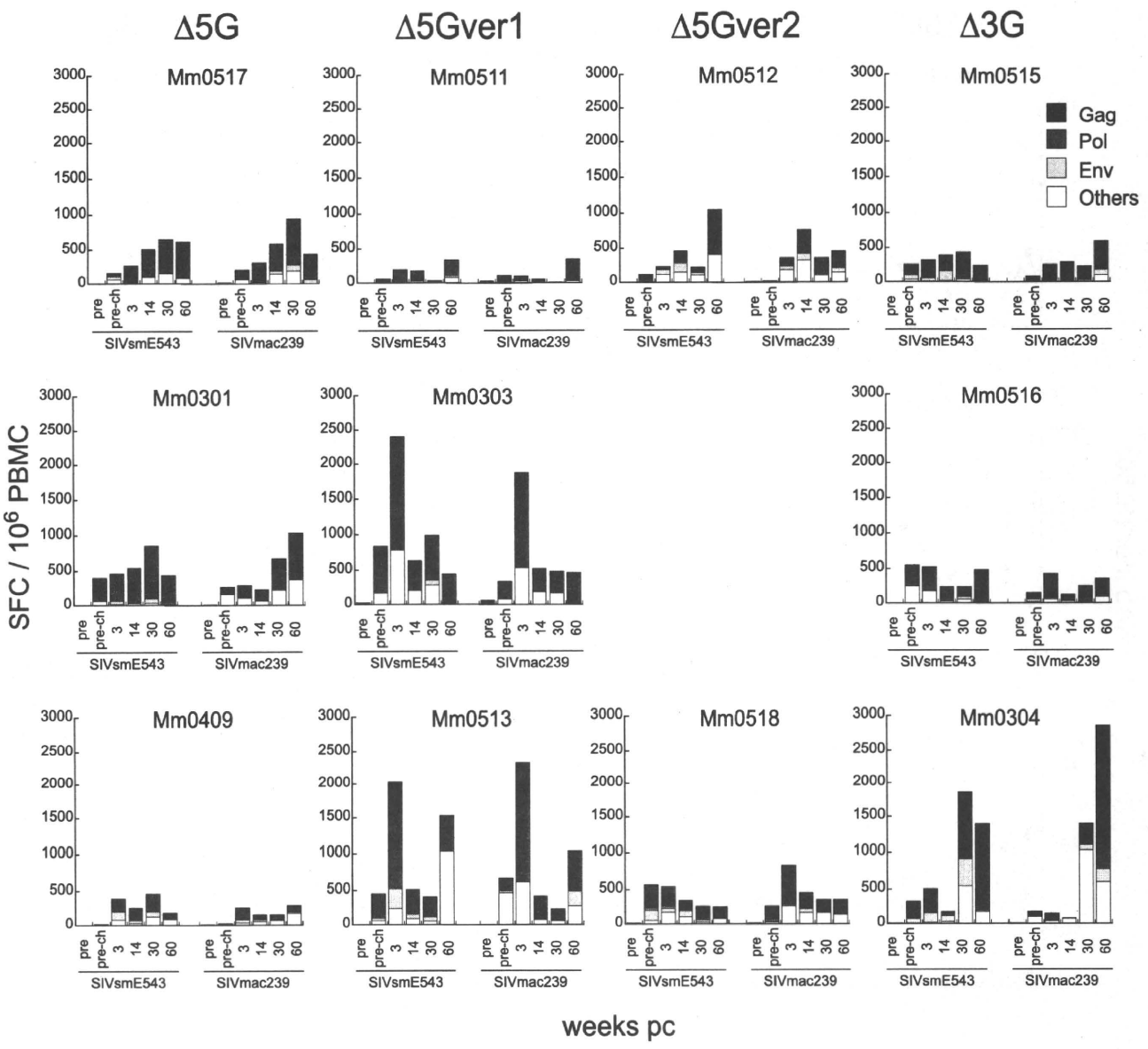

Figure 6. SIV specific cellular response in the vaccine recipients. SIV specific T cells were stimulated with overlapping peptides encompassing the viral proteins (Gag, Pol, Env, and Others: Vif, Vpr, Vpx, Tat, Rev, and Nef) of SIVmac239 and SIVsmE543-3 and the number of spot forming cells (SFC) per 10^6 PBMC determined utilizing the IFN-γ ELISPOT assay. The PBMC samples analyzed for the responses included those collected pre-vaccination, pre-challenge (4–8 weeks prior to the challenge) and at 3, 14, 30 and 60 weeks pc.
doi:10.1371/journal.pone.0011678.g006

from plasma RNA shown in Table 2 and plasma RNA from naïve control animals (the time-points chosen for analysis were shown by arrowheads in Figs. 4A and C). The newly replicating viruses were now found to possess restored numbers (>22) of N-glycosylation sites (Fig. 8). Mutation associated with N-glycosylation in gp120 of SIVsmE543-3 was a common feature of the SIV that we detected in non-controllers regardless of the occurrence of the recombination events. Accordingly, the viruses detected at later time-points had increased number of N-glycosylation sites compared with those from earlier time-points. For virus sequences that included 23 to 25 sites, additional N-glycosylation sites were acquired by single point mutations (Fig. 8). Of note, the addition of N-glycosylation sites preferentially occurred in the following three regions: V1, between V2 and the C-loop and V4 (Fig. 8). We also found a viral sequence with 2 additional N-glycosylation sites that reside within these hotspots in one of the naïve control animals (Mm0626) (Fig. 8). These results indicate that mutations associated with glycosylation of gp120 were associated with persistent viral replication during the chronic phase in all of the non-controllers and one of three naïve controls. These data suggest that glycosylation plays a prominent role in optimizing fitness and/or evasion from vaccine-induced host responses in these viruses.

Discussion

In this study, we examined whether reduction of glycosylation on viral spikes would allow for more ready access for host immune responses and thus provide for a new type of live attenuated vaccine, which would induce more robust anti-viral immune response and protect outbred rhesus macaques, against heterologous virus challenge. To evaluate the influence of allelic differences in host genetic properties on the efficacy of the

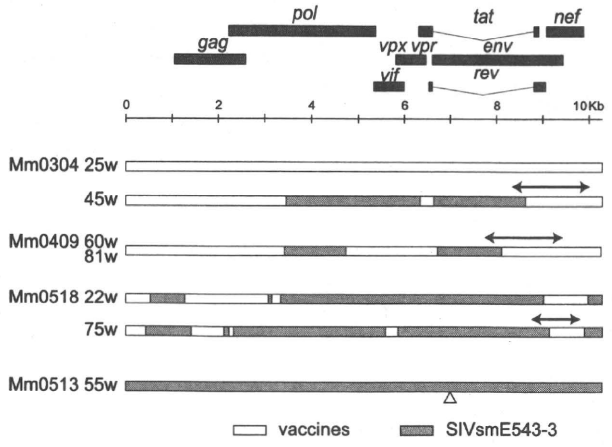

Figure 7. Recombination between vaccine and challenge virus. Nucleotide sequences of SIV fragments amplified by sets of nested PCR using primers based on SIVsmE543-3 or SIVmac239 to cover the entire SIV genome except the 5′ and 3′ terminal sequences (~100 bp). Representative sequences from integrated results of multiple sequences of PCR fragments are shown. The sequences detected in Mm0304 at 25 weeks pc were the vaccine virus sequences (top bar indicated by open box) and the sequences detected in Mm0513 at 55 weeks pc were the challenge virus sequences (bottom bar indicated by grey box) except in the case of the latter which contained a 9 bp deletion shown by triangle. Other represented sequences were recombinant viruses between vaccine (open boxes) and SIVsmE543-3 (gray boxes). Lines with arrowheads indicate the sequences that were targeted for nested PCR to quantify the recombinant viruses and SIVsmE543-3 in the non-controllers.
doi:10.1371/journal.pone.0011678.g007

vaccine, we used rhesus macaques with defined MHC-I and -II genes. Irrespective of differences in MHC genotypes, following a transient primary infection in approximately half of the vaccine recipients, all 11 vaccinated animals suppressed the acute-phase viral replication below the level of detection between 4 and 10 weeks pc (Figs. 4 B, C and D). After 10 weeks pc, containment of challenge virus infection diverged in two ways: 1) the majority (7 of the 11) of the vaccinated animals continued to control heterologous virus for more than 80 weeks pc without the contribution of elite MHC alleles previously associated with spontaneous CD8+ T cell mediated control of SIV replication in chronic SIV infection [6,7,22] (Fig. 4 B); 2) however, 4 of the vaccinated animals showed re-activation of SIV replication (Fig. 4 C) and three eventually developed AIDS. These results demonstrate that the host responses induced by these vaccines are capable of protecting from heterologous challenge virus at least during a limited period shortly after the challenge (10 weeks) irrespective of the inherited genetic properties of the host. However due to quantitative and/or qualitative changes in the protective response during the chronic-phase, viruses overcome the protective host response and most likely evolve viral diversity that is resistant to virus specific immune responses.

Table 2. SIVsmE543-3 derived and recombinant viruses in non-controllers[a].

Vaccinee	Weeks pc	SIVsmE543-3	Recombinants
Mm0304	60	0	320
	80	0	1600
Mm0409	60	0	200
Mm0518	60	3	60
	75	3	200

[a]Viral RNA in plasma was converted to cDNA, serially diluted and subjected to nested PCR to quantify SIVsmE543-3 and the recombinant viruses between the vaccine and SIVsmE543-3. Frequencies of SIVs were estimated as the total viral sequences detected by nested PCR using cDNA synthesized from 0.128 ml of plasma.
doi:10.1371/journal.pone.0011678.t002

The pathogenic viral replication was associated with the emergence of viruses that were recombinants between the vaccine virus and the challenge virus SIVsmE543-3 and was associated with an increased glycosylation of viral spikes (Figs. 7 and 8). On the other hand, a significant number of the vaccinated animals controlled the infection with SIVsmE543-3 not only during the primary-phase but also during the chronic-phase (Fig. 4 B). We speculate that the properties of the recombinants might be essential for these viruses to circumvent the protective responses in these three vaccinees, which were able to control both the vaccine and the challenge viruses. Interestingly, the recombinant viruses shared the common part of Pol and Env from SIVsmE543-3 and that of Gag and Nef from the vaccine viruses (Fig. 7). The complexity of the recombination patterns between vaccine and challenge viruses suggest that repeated events of super-infection with both viruses replicating concurrently must occur to allow for recombination to occur. Subsequently, only a few viruses that succeeded in evading vaccine-induced host responses have managed to replicate to substantial levels in these non-controllers. These viruses are assumed to have acquired distinctive properties conferred by the chimera structures that make them different from the original vaccine and challenge viruses. Viral spikes derived from SIVsmE543-3 with increased glycosylation are likely essential for these properties, since all of the SIV sequences examined at later time points had more than 23 N-glycosylation sites in gp120 of SIVsmE543-3 (Fig. 8). Although the changes of N-glycosylation sites in gp120 of SIV/HIV have been previously reported to be associated with escape from neutralizing antibody response [7,19,20], we did not detect any significant titers of neutralizing antibodies against the challenge virus in any of the 11 vaccinees (Fig. 5). Thus, modification of N-glycosylation sites in these viruses might play a role in inducing anti-viral host responses other than neutralizing antibody responses, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or such modifications might lead to altered viral properties or "viral fitness" such as tissue/cell tropism, replication levels, and/or stability in these animals.

It remains to be elucidated why an established immunity capable of containing the viral burst during acute infection gradually loses its grip over the virus, allowing for the generation of these mutant viruses. Select genetic properties required for prevention of emergence of escape mutants and/or viruses with altered fitness able to overcome vaccine-induced protective host responses might be lacking in these four animals. Indeed, MHC I allele A1*0560202 and the ones associated with MHC II haplotype 89002-p such as A1*01807 and others were identified only in non-controllers (File S1). Similarly, MHC I alleles such as A1*0040102, A1*11001, A1*03202 were indentified in controllers but not non-controllers suggesting immune responses regulated by MHC genes such as CTL and NK via KIR related mechanisms might play a role in viral control during the chronic-phase. Our attempts to identify immunological correlates of protection suggested that magnitude of overall T cell responses could not account for either the marked containment of the infection during the acute-phase or the different outcome of the infection between controllers and non-controllers during the chronic-phase. Nevertheless, the fact that Gag-specific T cells constitutes more than

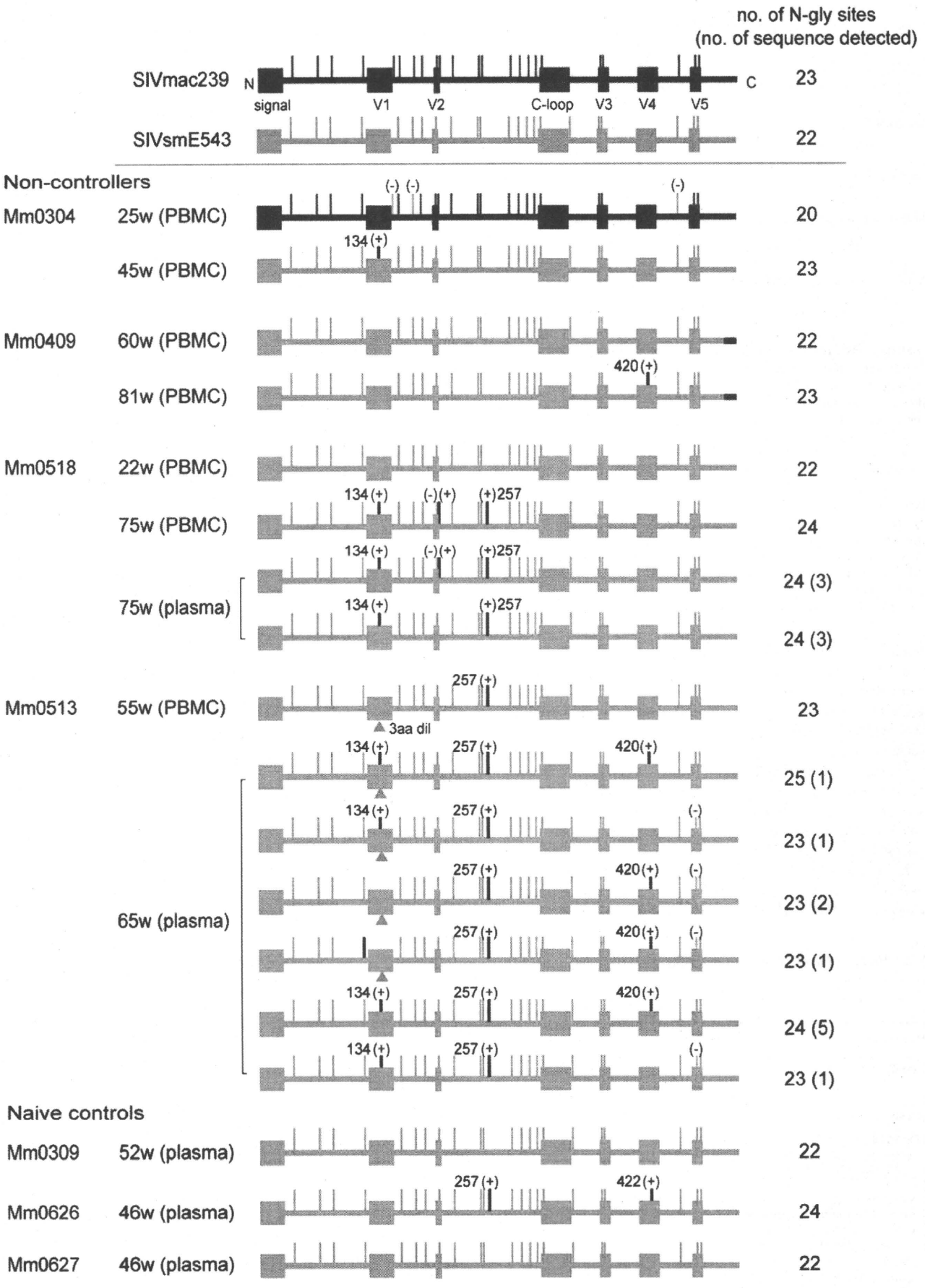
no. of N-gly sites
(no. of sequence detected)
SIVmac239
N
C
signal
V1
V2
C-loop
V3
V4
V5
23
SIVsmE543
22
Non-controllers
Mm0304
25w (PBMC)
(-) (-)
(-)
20
45w (PBMC)
134 (+)
23
Mm0409
60w (PBMC)
22
81w (PBMC)
420 (+)
23
Mm0518
22w (PBMC)
22
75w (PBMC)
134 (+)
(-)(+)
(+)257
24
75w (plasma)
24 (3)
24 (3)
Mm0513
55w (PBMC)
257 (+)
3aa dil
23
65w (plasma)
420(+)
25 (1)
23 (1)
23 (2)
23 (1)
24 (5)
23 (1)
Naive controls
Mm0309
52w (plasma)
22
Mm0626
46w (plasma)
422 (+)
24
Mm0627
46w (plasma)
22

Figure 8. N-glycosylation sites in gp120 of the emerged viruses. The putative N-glycosylation sites (vertical bars) in gp120 were analyzed based on sequencing data from PBMC and plasma samples obtained from non-controllers. The red lines indicate SIVmac239 or vaccine virus sequences, and blue lines indicate SIVsm534-3 sequences. The notations (−) and (+) indicate the loss and addition of N-glycosylation site respectively. The triangle sign indicates a 3 amino acid deletion located within the V1 region found in the Mm0513. The numbers shown besides (+) denote the amino acid sequence numbers for hotspots of addition of N-glycosylation sites based on SIVsmE543-3 Env amino acid sequence (accession no. U72748). The total number of N-glycosylation sites found in each sequence was shown on the right, and the numbers in parenthesis indicate the number of sequences detected by PCR.
doi:10.1371/journal.pone.0011678.g008

50% of the repertoire of SIV specific T cells in the vaccinees (Fig. 6) suggest an important role for these responses in the containment of the heterologous virus infection. The magnitude of such responses may also be critical during acute infection illustrated by the association of strong acute cellular response and rare detection of recombinant virus in one vaccinee Mm0513, that prevented disease progression (Figs. 4 C and 6).

Efficacy of a live attenuated vaccine against heterologous virus has also been studied using nef gene deleted mutants including SIVmac239Δnef [23] and SIVmac239Δ3 [24] as live attenuated vaccines. It is difficult to compare those results with results of the studies reported herein obtained under similar but not exactly the same conditions. However, a number of differences between the use of SIVΔnef and the studies reported herein were noted: First, the control of acute-phase viral infection occurred in the vaccinees with MHC I alleles associated with elite controller, Mamu B*08 and B*17 in the Δnef vaccine study. In contrast, our study indicated control of the primary infection in all of the vaccinees irrespective of the diversity of MHC genotypes. Second, the containment of the challenge virus in the chronic-phase also appeared to be much less prominent in these two studies. These differences might stem from the intrinsic properties of the two types of live attenuated SIV. We have previously reported that SIVmac239Δnef and SIVmac239 with a functional *nef* gene replicate preferentially in B cell areas and T cell areas, respectively, in peripheral lymph nodes during primary infection [17]. On the other hand, Δ5G replicates preferentially in CD4+T cells in intestinal effector sites such as lamina propria, whereas the wild-type SIVmac239 replicates in CD4+T cells in inductive sites such as T cell areas of secondary lymphatic tissues. These subtle differences of tissue and cell tropism suggests that the mechanisms of attenuation may differ between Δ5G and SIVmac239Δnef and may further explain why the latter is likely more pathogenic than the former. In addition, the differences in the susceptibility of the macaques to SIV, estimated by the magnitude of peak VL during primary infection and set point VL, could be another factor that influenced the results of the studies. In nonhuman primate model for AIDS, the properties of SIV strains and the origin of macaques appear to affect the results and interpretation of the data from the experiments. Judged from previous studies from a number of other laboratories including ours that have utilized Burmese [8,16,17] and Indian macaques [6,25,26,27] respectively, Burmese rhesus macaques infected with SIVmac239 tend to have lower set point VL and require more time to develop AIDS than Indian rhesus macaques. Thus, these differences might have allowed us to discover potent protective host responses against heterologous virus elicited by a deglycosylated live-attenuated vaccine. On the other hand, this study also demonstrates that Burmese macaques were more susceptible to SIVsmE543-3 than SIVmac239 (Figs. 3 and 4 A). In fact, these results indicate that SIVsmE543-3 and SIVmac239 might form an excellent model of heterologous challenge virus and a template virus to create vaccine viruses. These results also underline that macaque susceptibility to SIV might be more SIV strain specific than previously considered.

In summary, we report here for the first time, the induction of potent protective immunity against heterologous challenge by live attenuated SIV in macaques with a diverse MHC genetic background. Our system provides a unique and robust experimental paradigm for defining the potential immunological correlates of protection, assessing cross-subtype protection and designing HIV vaccines. However, emergence of pathogenic revertants from live attenuated SIVs by spontaneous mutations as well as by recombination has often been encountered in macaque AIDS models [23] [28] and certainly during our study. Thus, while a live vaccine strategy is clearly not a viable approach to actual HIV vaccine development, much can be learnt with regards to the mechanisms involved. As noted above, continuous stimulation of the host immune system by persistently infected vaccine virus at low levels may be a key factor for maintaining protective immunity not only against homologous but also heterologous SIV over a long period. We believe that creating such a condition, for instance, by a virus vector capable of establishing a persistent infection may be one strategy that may lead to the development of an effective vaccine against HIV. Minimally, the heterologous virus challenge model described herein provides a powerful tool to attempt to identify the potential mechanisms that lead to protective versus non-protective immunity. We reason that such events are likely to have occurred during the acute phase of "vaccine" virus replication which sets the course for the eventual response of the animals to the heterologous challenge virus. A detailed study of events that transpire during the acute infection period may provide unique insights on this issue.

Materials and Methods

Mean distance of amino acid sequences of HIV-1 group M subtypes and amino acid differences between SIVmac239 and SIVsmE543-3

The complete genome sequence alignments consist of 368 HIV-1 isolates (59 subtype A, 71 subtype B, 148 subtype C, 39 subtype D, 6 subtype F1, 3 subtype F2, 6 subtype G, 3 subtype H, 2 subtype J, 2 subtype K, 15 CRF01_AE, and 14 CRF02_AG) as determined from HIV sequence database (http://www.hiv.lanl.gov/cgi-bin/NEWALIGN/align.cgi) were used for these analyses. The alignment data was coordinated with HXB2-LAI-IIIB. These data led to the identification of nine coding regions, as determined utilizing the MEGA4 software [**29**]. We estimated the number of amino acid differences per site from averaging the over all sequence pairs between and within each subtype or CRF, and also mean diversity. All results are based on the pairwise analysis of the sequences, and standard error estimates were obtained by a bootstrap procedure (500 replicates). All positions containing gaps and missing data were eliminated from the dataset. The amino acid comparisons in each viral protein between SIVmac239 (Genbank accession no. M33262) and SIVsmE543-3 (Genbank accession no. U72748) were analyzed by Clustal W (http://www.clustal.org).

Attenuated vaccine viruses and challenge virus

The molecular pathogenic clone of SIVmac239 [30] and its derived deglycosylated mutants used in this study are depicted in

Fig. 1. The Δ5G was derived by site-directed mutagenesis of an SIVmac239 infectious DNA clone so that the asparagine residues for the N-glycosylation sites at aa 79, 146, 171, 460 and 479 in gp120 were converted to glutamine residues [2,3]. The Δ5G-ver1, Δ5G-ver2 and Δ3G were also constructed by site-directed mutagenesis from the series of deglycosylated mutants reported previously [2]. The stocks of deglycosylated mutants were prepared by DNA transfection of respective proviral DNAs into 293T cells. The stock of SIVsmE543-3 was prepared as previously described [4]. These virus stocks were propagated in phytohemagglutinin-stimulated peripheral PBMC from rhesus macaques as previously described [3,16].

Animals

Juvenile rhesus macaques originating from Burma were used following negative results of screening for SIV, simian T-cell lymphotropic virus, B virus, and type D retrovirus infection prior to study inception. All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare as outlined by National Institute of Infectious Diseases and National Institute of Biomedical Innovation, Japan. Full details of the study were approved (Approval number: 507006) by National Institute of Infectious Diseases Institutional Animal Care and Use Committee in accordance with the recommendations of the Weatherall report. Early endpoints are adopted including frequent monitoring of viral loads and immunological parameters, and humane euthanasia is conducted once any manifestation of clinical AIDS or signs of fatal disease is noted.

Vaccination and challenge infection

Three animals per group were intravenously inoculated with 100 $TCID_{50}$ of either of 4 deglycosylation mutants (Δ5G, Δ5G-ver1, Δ5G-ver2 and Δ3G) as shown in Fig. 1. At 40 weeks post infection, 4 SIV-infected animals: Mm0301 (Δ5G), Mm0513 (Δ5G-ver1), Mm0307 (Δ5G-ver2), Mm0304 (Δ3G) and three naive animals (Mm0608, Mm0521, and Mm0522) were intravenously inoculated with 1000 $TCID_{50}$ of SIVmac239 for purposes of homologous virus challenge studies.

To examine the efficacy of the live attenuated vaccine against heterologous virus, 11 vaccinees were intravenously inoculated with 1000 $TCID_{50}$ of SIVsmE543-3.3 as follows: Mm0517 (Δ5G), Mm0511 (Δ5G-ver1), and Mm0512 (Δ5G-ver2) were challenged at 50 weeks post vaccination with the deglycosylation mutant; Mm0409 (Δ5G), Mm0303 (Δ5G-ver1), and Mm0518 (Δ5G-ver2) were challenged at 61 weeks post vaccination; Mm0515 (Δ3G) and Mm0516 (Δ3G) were challenged at 117 weeks post vaccination. 3 naïve animals (Mm0309, Mm0626, Mm0627) were infected with SIVsmE543-3 as vaccine-naïve controls. Furthermore, three of SIVmac239-challenged animals, Mm0301, Mm0513 and Mm0304 (Mm0307 died with a SIV-infection-unrelated cause) were re-challenged with SIVsmE543-3 at 117 weeks post vaccination and 77 weeks post SIVmac239 challenge.

Plasma viral load measurements

SIV infection was monitored by measuring the plasma viral RNA load using a highly sensitive quantitative real-time RT-PCR. Viral RNA was isolated from plasma samples from infected animals using MagNA PureCompact Nucleic Acid Isolation Kit (Roche Diagnostics). Real-time RT-PCR was performed by using QuantiTec Probe RT-PCR kit (Qiagen) and Sequence detection system SDS7000 (Applied Biosystems). To detect SIVmac239 gag and SIVsmE543-3 gag separately, primers and probe sets were synthesized as follow; SIVsmE543-3 gag specific primers: 5′- FAM-GCAGAGGAGGAAATTACCCAGTGC-3′, 5′-CAATTTTACCCAAGCATTTAATGTT- TAMRA- 3′ and probe 5′-TGTCCACCTACCCTTAAGTCCAA-3′, SIVmac239 specific gag primers: 5′-GCAGAGGAGGAAATTACCCAGTAC-3′, 5′-CAATTTTACCCAGGCATTTAATGTT-3′ and probe 5′-FAM-TGTCCACCTGCCATTAAGTCCCGA-TAMRA-3′. These primers and probes do not cross-react with SIVmac239 RNA and SIVsmE543-3 RNA. The detection sensitivity of plasma viral RNA by this method was calculated to be 100 viral RNA copies per ml of plasma.

Sequencing of SIV RNA and proviral DNA

Viral RNA was isolated using MagNA PureCompact Nucleic Acid Isolation Kit (Roche Diagnostics) and cDNA was synthesized with two-step qRT-PCR kit (Invitrogen). PBMC from vaccine recipients were suspended with lysis buffer (10mM Tris 0.5% NP-40 and 0.5% Tween20) with Proteinase K (200 mg/ml), and incubated at 55°C for 1 hour, then heat-inactivated at 95°C for 5 min. Serial 10-fold diluted cDNA or cell lysate was subjected to nested PCR with the Ex-Taq PCR kit (Takara, Tokyo, Japan) with the following condition: 1 cycle of 97°C for 1 min. and then 25 cycles of amplification (94°C for 30 s, 55°C for 30 s, 72°C for 2.5 min) and 72°C for 10 min. and then 4°C for 5 min. Primers were designed to target the several overlapping sequences spanning the open reading frames of SIVmac239 or SIVsmE543-3 as shown in File S1. Positive PCR products were sequenced by using BigDye terminator cycle sequencing kits (Applied Biosystems) and analyzed by using ABI3100 or ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequences were assembled using ATGC version 4.2 (Genetyx Corporation).

SIV specific T cell responses

The T cells in the animals were examined for virus specific cellular response against the vaccine virus and the challenge virus by using pooled peptides covering overlapping sequences of all viral proteins of SIVmac239 and SIVsmE543-3 respectively. Briefly, cryopreserved PBMC were thawed, resuspended at 2×10^6 cells/ml in R10 (RPMI1640 supplemented with 10% heat-inactivated FCS, 55 μM 2-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin), and rested for 2 h at 37°C. The cells were washed and aliquots of 10^5 cells were stimulated with each pool of peptides at a final concentration of 2 mg/ml in an anti-IFN-g Ab-coated plate overnight. ELISPOT assay for the detection of IFN-g secreting cells were performed using a commercial ELISPOT kit (U-CyTech Bioscience). Peptides based on sequences of SIVmac239 viral proteins were synthesized by the Microchemical Facility, Emory University School of Medicine, Atlanta, GA, USA. Peptides based on the sequences of SIVsmE543-3 viral proteins were synthesized by Sigma-Aldrich Japan.

Neutralization assay

Virus neutralizing antibodies were tested according to a protocol using CEMx174/SIVLTR-SEAP cells [31] as described previously [3]. Serially diluted heat-inactivated plasma was tested for inhibition of the corresponding vaccine virus or the challenge virus (SIVsmE543-3) in CEMx174/SIVLTR-SEAP cells. SEAP activity in the culture supernatant was assayed using a commercial SEAP reporter gene assay chemiluminescent kit (Roche Diagnostics).

Statistical analysis

Correlation analysis was performed using Spearman's non-parametric rank test and Mann-Whiney 'U' test by using Graph

pad Prism 4.0 software. Correlations were considered statistically significant when P values were <0.05.

DNA sequence data deposition

The SIV sequences reported in this paper have been deposited in the DNA Data Bank of Japan (accession nos. AB553915 to AB554013).

Supporting Information

File S1

Found at: doi:10.1371/journal.pone.0011678.s001 (0.28 MB PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: CS AK MM YS NY KM. Performed the experiments: CS SW TN EK NU KU HS KM. Analyzed the data: CS SW TN EK TS NU KU HS KM. Contributed reagents/materials/analysis tools: SO VH. Wrote the paper: CS FV AAA YN KM.

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ORIGINAL ARTICLE

Validation of recombinant Sendai virus in a non-natural host model

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We have previously shown that recombinant Sendai virus (SeV) vector, derived from murine parainfluenza virus, is one of the most efficient vectors for airway gene transfer. We have also shown that SeV-mediated transfection on second administration, although reduced by 60% when compared with levels achieved after a single dose, is still high because of the efficient transfection achieved by SeV vector in murine airways. Here, we show that these levels further decrease on subsequent doses. In addition, we validated SeV vector repeat administration in a non-natural host model, the sheep. As part of these studies we first assessed viral stability in a Pari LC Plus nebuliser, a polyethylene catheter (PEC) and the Trudell AeroProbe. We also compared the distribution of gene expression after PEC and Trudell AeroProbe administration and quantified virus shedding after sheep transduction. In addition, we show that bronchial brushings and biopsies, collected in anaesthetized sheep, can be used to assess SeV-mediated gene expression over time. Similar to mice, gene expression in sheep was transient and had returned to baseline values by day 14. In conclusion, the SeV vector should be strongly considered for lung-related applications requiring a single administration of the vector even though it might not be suitable for diseases requiring repeat administration.
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Keywords: Sendai virus; cystic fibrosis; lung; gene transfer; sheep

INTRODUCTION

Sendai virus (SeV), a murine paramyxovirus, is one of the most efficient viral vectors for airway gene transfer.[1,2] SeV carrying the cystic fibrosis (CF) transmembrane conductance regulator cDNA is able partially to correct the characteristic CF transmembrane conductance regulator-dependent chloride transport defect in the nasal epithelium of CF knockout mice.[3] Several aspects of SeV biology may explain the high gene transfer efficiency into airway epithelial cells. Moreover, SeV uses cholesterol and sialic acid as receptors and both are present on the surface of most cell types. Further, SeV requires short contact time with the target cell for internalization, and replicates in the cytoplasm of transduced cells, circumventing the nuclear membrane barrier. In mice gene expression is transient, with peak expression approximately 48 h after transfection, generally returning to baseline values within 2 weeks of transfection.[1] A transmission-incompetent SeV vector has been developed by deleting the F-protein, which is essential for cellular entry of the viral genome (ΔF/SeV).[4] Moreover, this modification does not reduce transfection efficiency of the virus.[1,4]

In general the level of transgene expression achieved from repeat delivery of a viral vector is greatly reduced when compared with that from a single dose due to induction of effective immune responses in the recipient.[5,6] We have previously shown that SeV-mediated gene expression is reduced by 60% on second dose, and that tolerization of mice against immune-dominant SeV epitopes does not improve efficacy.[7] However, given the extremely high transfection efficiency of SeV, the levels of gene expression achieved after repeat administration may still be of sufficient therapeutic value, if retained on subsequent administrations. Here, we assessed SeV-mediated transfection efficiency after three doses of the virus and compared residual levels of gene expression with that achieved using plasmid DNA complexed to the cationic lipid GL67A, currently being used in a clinical trial by the UK CF Gene Therapy Consortium (http://www.cfgenetherapy.org.uk/).

The vast majority of repeat administration studies have been performed in mouse inbred strains, such as Balb/C and C57BL/6. Given that SeV has a natural tropism for the murine lung leading to pneumonia, we were concerned that assessment of repeat administration in mice may not be representative of non-natural host models, including man. The greater degree of similarity in size, structure and physiology between the sheep and human lung led us to develop sheep as an intermediate model for airway gene transfer,[8] to bridge the gap between studies in rodents and the clinic. Here, we have also assessed the transduction efficiency of both single and repeat SeV vector administration in the ovine lung.

RESULTS

Levels of gene expression after repeat administration of ΔF/SeV with short-term intervals to mouse lung are similar to non-viral gene transfer

We have previously shown that SeV-mediated transfection on second administration, although reduced by 60% when compared with levels

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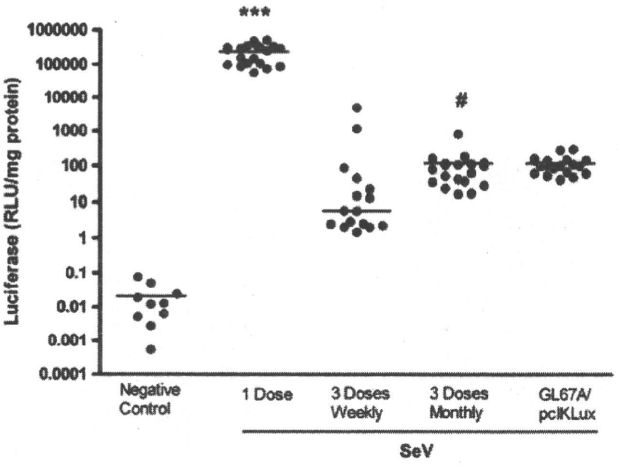

Figure 1 Repeat administration of ΔF/SeV in mouse lung. Murine lungs were transfected with three doses (10^8 CIU/mouse/dose) of transmission incompetent ΔF/SeV at either weekly or monthly intervals by nasal sniffing. To circumvent generation of anti-reporter gene immune responses a vector without a reporter gene (ΔF/SeV-empty) was administered for the first two doses. A vector carrying a luciferase reporter gene (ΔF/SeV-luc) was administered as the third dose and luciferase (luc) expression in lung was quantified two days after administration (n=16–19 mice/group). Luc expression was compared with mice receiving only a single dose of virus (n=20), mice transfected with the non-viral vector GL67A complexed to pCIK-Lux (n=19) and PBS-treated negative controls (n=10). Each symbol represents one mouse. The horizontal bar indicates group mean. ***$P<0.001$ compared with single-dose cohort. #$P<0.05$ compared with untransfected mice.

achieved after a single dose,[7] is still high because of the efficient transfection achieved by SeV vector in murine airways. Here, we assessed whether these residual levels are maintained on subsequent dosing. Murine lungs were transfected with three doses (10^8 cell infectious unit (CIU)/mouse/dose) of transmission-incompetent ΔF/SeV at either weekly or monthly intervals by nasal sniffing. To circumvent generation of anti-reporter gene immune responses, a vector without a reporter gene (ΔF/SeV-empty) was administered for the first two doses. A vector carrying a luciferase reporter gene (ΔF/SeV-luc) was administered as the third dose, and luciferase (luc) expression was quantified 2 days after administration (n=16–19/group). Luc expression was compared with mice receiving only a single dose of virus (n=20), mice transfected with the non-viral vector GL67A complexed to pCIK-Lux (n=19) and phosphate-buffered saline (PBS)-treated negative control mice (n=10). Gene expression after 3 weekly or monthly, doses was significantly ($P<0.001$) reduced by 3 to 4 logs compared with mice receiving only one dose, but was significantly ($P<0.05$) higher than the negative controls (Figure 1). Residual expression levels after three doses of ΔF/SeV were not significantly different to non-viral transfection (Figure 1).

SeV viability in the Trudell AeroProbe catheters is suitable for *in vivo* administrations

We next assessed transfection efficiency and repeat administration in sheep for which the virus does not have a natural tropism. In preparation for the *in vivo* sheep experiments, we first assessed ΔF/SeV viability in a Pari LC Plus nebuliser, which we routinely use for non-viral gene transfer in sheep. We assessed viability by either collecting ΔF/SeV aerosol at the end of the Pari LC Plus mouth-piece (as used in man) or at the end of an endotracheal tube (as used for sheep nebulization studies). ΔF/SeV viability was reduced to less than 1% in the 'mouth-piece' set-up and dropped to less than 0.01% when connected to an endotracheal tube (three independent experiments). This delivery method was, therefore, not used in any further study.

We next assessed virus viability in a polyethylene catheter (PEC), which can be fed through the biopsy channel of a bronchoscope and used to administer virus as a bolus into specific lung segments, and a Trudell AeroProbe catheter, which can be positioned within the trachea or bronchi and generates an aerosol at the tip. Virus viability was 100 and 49 ± 3% in the PEC and AeroProbe, respectively (three independent experiments/condition).

PEC and AeroProbe administration of ΔF/SeV-LacZ leads to wide-spread β-gal expression in the sheep lung but the distribution pattern varies

ΔF/SeV-LacZ (3.4×10^9 CIU/sheep. in 5 ml, n=3 sheep) was first administered by PEC as a bolus into a single lung segment. Gene expression was visualized with X-gal staining 48 h after transfection. High level, but patchy, transfection was seen in the lung possibly due to uneven vector distribution and pooling of the bolus volume compatible with catheter-based delivery (Figure 2a). Using segmental instillation the adjacent lung segments showed no evidence of transgene expression (Figure 2b).

We next administered ΔF/SeV-LacZ using the Trudell AeroProbe to either a single lung segment (10^{10} CIU per sheep in 5 ml, n=1) or the whole lung (4×10^{10} CIU per sheep in 24 ml, n=1). When directed into a single segment we observed spill over to the adjacent segment on the same (Figures 2c and d), but not to the contralateral side, of the lung (data not shown). This is likely due to a combination of pooling of the aerosol following impaction on the airway walls close to the tip of the catheter and the fact that the bronchoscope is not wedged in the airway. Figures 2e and f demonstrates deposition of aerosol on airway bifurcations and, interestingly, within a sub-mucosal gland and duct.

With the Trudell AeroProbe positioned in the trachea we observed more even, wide-spread distribution throughout the lung and less of a pooling effect, likely due to the AeroProbe tip not being so close to airway walls (Figure 2g). The Trudell AeroProbe was, therefore, used for subsequent experiments.

Infectious virus shedding does not occur after ΔF/SeV transfection

Infectious virus shedding after sheep transfection would be a concern, because both the environment and the operators would require protection from exposure to virus. We, therefore, determined whether virus shedding had occurred. Nasal and oral swabs, bronchoalveolar lavage fluid and lung tissues were collected 48 h after transfection with ΔF/SeV-LacZ (10^{10} CIU in 24 ml per sheep, n=5 sheep). Three plates were analyzed per sample per sheep. Virus spiked positive controls and untreated samples were analyzed in parallel. A small number of Xgal-positive cells (one to three cells per plate) were detected in the collected fluids and tissues of transfected sheep, but these were also present in the untreated controls. In a second group of animals (n=3) sampled *in vivo* 48 h post-administration we saw no X-gal-positive cells from oral or nasal swabs, broncho-alveolar lavage fluid or serum samples. We conclude that infectious virus shedding does not occur at a detectable level at 48 h post-transfection.

Bronchial brushings and biopsies can be used to assess SeV-mediated transfection

Although LacZ is a useful reporter gene to visualize gene expression histologically, we were not certain whether β-gal expression would be

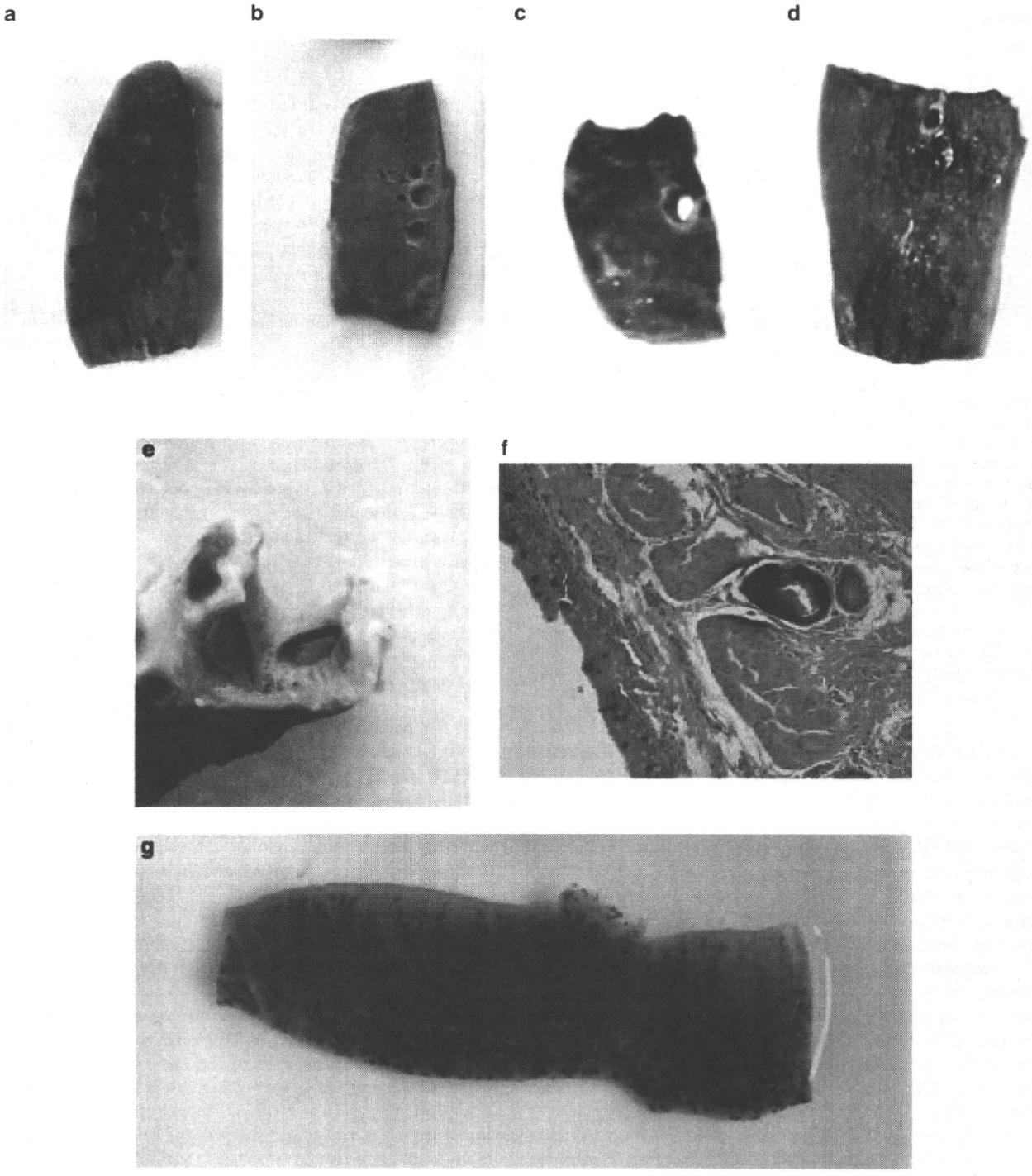

Figure 2 β-gal expression in sheep lung 2 days after transfection with ΔF/SeV-LacZ delivered with either a polyethylene catheter (PEC) or the Trudell AeroProbe. ΔF/SeV-LacZ was administered to a single segment of the sheep lung with a PEC (3.4×10^9 CIU/sheep in 5 ml, n=3 sheep). High level, but patchy, gene expression was seen in the treated segment (**a**), but not in the adjacent segment (**b**). ΔF/SeV-LacZ was administered to a single segment using the Trudell AeroProbe (10^{10} CIU/sheep in 24 ml, n=1). Gene expression was visible in the treated, as well in the adjacent segment (**c**, **d**). In addition β-gal expression was detected on airway bifurcations and within a sub-mucosal glands (**e**, **f**). ΔF/SeV-LacZ was administered to a whole lung using the Trudell AeroProbe (4×10^{10} CIU/sheep in 24 ml, n=1) and wide-spread even distribution throughout the lung was observed (**g**). Representative images are shown.

quantifiable in bronchial brushings and biopsies, used to assess duration of gene expression *in vivo*. We, therefore, collected bronchial brushings (n=4/sheep) and biopsies (n=4/sheep) before transfection, then administered ΔF/SeV-LacZ (10^{10} CIU in 24 ml/sheep, n=3 sheep) and collected bronchial brushings and biopsies (n=4 each for one sheep, n=8 each for the other two sheep) and tissue pieces at necropsy 48 h post transfection. Using a chemiluminescent β-gal assay we were able to detect robust levels of β-gal in cell lysates prepared from

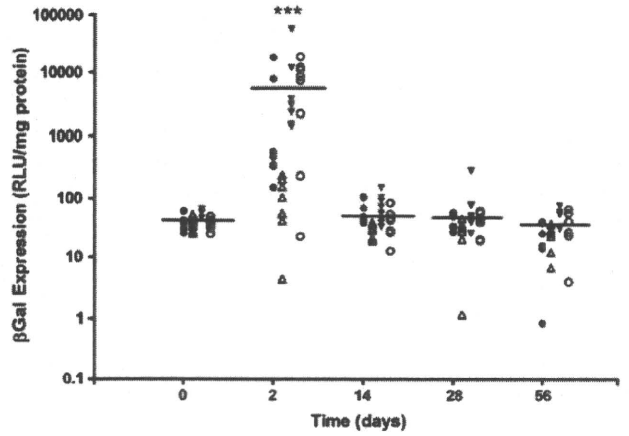

Figure 3 Duration of gene expression in sheep lung. Sheep were transfected with ΔF/SeV-LacZ (10^{10}CIU in 24 ml per sheep, n=4 sheep) and biopsies were collected 2, 14, 28 and 52 days after transfection (n=8/sheep/time point) to follow gene expression over time. Each symbol represents one biopsy. Horizontal bars indicate group mean. Individual sheep are marked as different symbols. ***$P<0.001$ compared with all other groups.

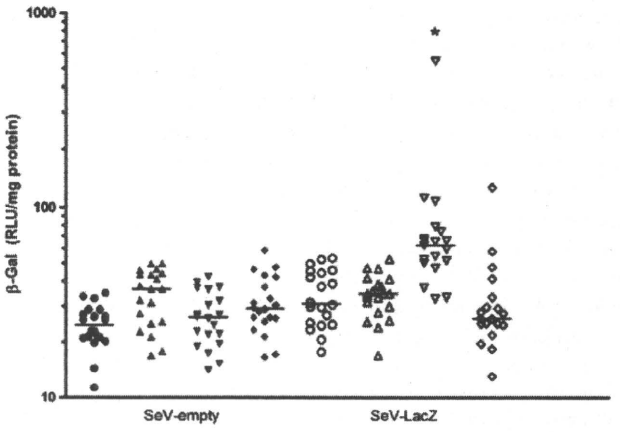

Figure 4 Repeat administration (3 doses monthly) of ΔF/SeV-LacZ in sheep. Sheep lung was transfected with three doses (10^{10}CIU/sheep/dose) of ΔF/SeV at monthly intervals. To circumvent generation of anti-reporter gene immune responses ΔF/SeV-empty was administered for the first two doses. ΔF/SeV-LacZ was administered as the third dose (n=4 sheep, open symbols) and β-gal expression was quantified in bronchial biopsies (n=20/sheep) 2 days after administration. A negative control group was treated with three doses of ΔF/SeV-empty (n=4 sheep, closed symbols). Each symbol represents one biopsy (n=19–20 biopsies/sheep). Horizontal bars indicate group medians. Individual sheep is marked as different symbol. *$P<0.05$ compared with all other sheep.

brushings and biopsies (brushings, pre: 66 ± 25, post: 7781 ± 6376 relative light unit per mg protein, $P<0.01$, biopsies, pre: 84 ± 40, post: 3074 ± 1551 relative light unit per mg protein, $P<0.001$). Levels of gene expression in biopsies and brushings taken from the same lobe correlated well (r^2=0.65, P=0.003). However, we were unable to reliably quantify β-gal expression in cell lysates prepared from tissue pieces, most likely due to the intensive red colour of the lysate interfering with the assay (data not shown). Thus, repeated sampling of bronchial brushings and biopsies should allow repeated analysis of gene expression over time *in vivo*.

ΔF/SeV gene expression in sheep is transient

We next administered a dose of ΔF/SeV-LacZ to each side of the lung with the AeroProbe positioned in the right and left main bronchi. Each side received a dose of 5×10^9 CIU in 7 ml (n=4 sheep) and bronchial biopsies were collected 2, 14, 28 and 56 days after transfection (n=8/sheep/time point) to follow gene expression over time by comparison with pre-treatment biopsies (n=6/sheep). Similar to mice, the expression was transient and had returned to baseline values by day 14 (Figure 3).

Reduced expression from repeat administration of ΔF/SeV in sheep

To determine the efficacy of repeat administration, sheep were transfected with three doses (10^{10} CIU/sheep/dose) of ΔF/SeV at monthly intervals. To circumvent generation of anti-reporter gene immune responses ΔF/SeV-empty was administered for the first two doses. ΔF/SeV-LacZ was administered as the third dose and β-gal expression was quantified in bronchial biopsies (n=20/sheep) 2 days after the final administration. A negative control group was treated with three doses of ΔF/SeV-empty (n=4 sheep/group). Although we detected some residual expression in one sheep ($P<0.05$), overall there was no evidence that re-administration of SeV into sheep is feasible (Figure 4). We, therefore, concluded that monthly re-administration of ΔF/SeV in sheep results in a similar loss of efficiency as we observed in mice. For comparison, GL67-mediated non-viral gene transfer does not lead to detectable levels of reporter protein expression in sheep bronchial biopsies or brushings (data not shown).

DISCUSSION

SeV and the modified transmission incompetent ΔF/SeV are one of the most efficient gene transfer agents for airway epithelial cells, but here we show that repeat administration (three doses at short-term intervals) into mice, which is the natural host, is inefficient. In addition, we optimized SeV-mediated gene transfer into sheep lung for which the virus has no natural tropism, achieving efficient transduction. However, as in mice, repeat administration was unable to achieve the high levels of gene transfer that could be demonstrated following a single dose.

Reports on repeat administration of adenovirus or adeno-associated virus to the lungs vary, with some studies reporting successful re-administration,[9–11] whereas other studies failed to detect gene expression after repeat administration.[6,12] Differences are likely due to different models being used, as well as different number of doses being administered. In some publications, single-dose controls are also missing, which makes an assessment of efficacy after repeated administration difficult.[10]

The number of repeat administrations (two vs three) is an important variable when performing and interpreting the efficacy of repeat-dose virus administration. Several population vaccination programmes, for example, human papillomavirus and hepatits A vaccine, require a three-dose schedule. In the context of virus-mediated gene transfer, it is feasible that the immune system may not be fully activated after only two doses of virus. Consequently, analysis of gene expression after additional doses is necessary before definite conclusions about repeat administration of viral vectors can be drawn. This is supported by our data. After a second dose of ΔF/SeV, expression is significantly reduced by 60 and >90% in mice[7] and sheep, respectively. However, because of the original extremely high gene transfer efficiency the residual gene expression after the second dose is still significantly higher than untransfected controls, or a single dose of non-viral vector. After subsequent doses, however, gene expression drops further leading to very low levels of residual gene expression. In mice these levels are similar to GL67A-

mediated expression and repeat administration of SeV does not, therefore, offer an advantage over the cationic lipid GL67A. The latter is the current 'gold-standard' lipid for airway transfection and is currently used by us in clinical trials for CF gene therapy. Moreover, repeat administration of ΔF/SeV into its natural host (mice) and non-natural host (sheep) models led to similar results.

This study also assessed our understanding of ΔF/SeV vector delivery to the lungs of large animal models. Thus, (1) we showed that, ΔF/SeV is not stable in the commonly used Pari-LC Plus jet nebuliser. Although this nebuliser is suitable for aerosolization of lipid/DNA complexes[13] and non-enveloped adeno-associated virus[14] and has been used in gene therapy clinical trials,[15,16] the shear forces generated appear to destroy the enveloped SeV. The latest generation of vibrating mesh-based single-pass nebulisers, such as the Pari eFlow, will need to be assessed for the delivery of enveloped virus.

(2) ΔF/SeV was stable in polythylene catheters (100% stability) and the Trudell AeroProbe (approximately 50% stability). The decrease in viability observed with the Aeroprobe was not unexpected as only 40% of viable particles were recovered after aerosolization of helper-dependent adenovirus vectors through catheters rated at 8 μm, rising to 80% with 15 μm, respectively.[17]

(3) In addition, we were able to compare virus distribution after catheter-based segmental administration and Trudell AeroProbe aerosolization. Delivery by instillation results in patchy expression in the lungs likely due to the uneven distribution and pooling effect from the delivery of a bolus. The AeroProbe catheter has the advantage that it generates an aerosol at the tip of the catheter. This results in a cone-shaped particle stream of aerosol visible to the eye even when viewed through the bronchoscope camera. To target an individual segment, we positioned the Aeroprobe in a small bronchus (with an approximate diameter of <5 mm) leading to that segment and observed that a significant proportion of the aerosol stream impacted on the airway wall close to the catheter tip and collected in a puddle. This resulted in a transgene expression pattern similar to that observed with the instillation delivery and significant spill-over to the adjacent lung segment.

In contrast, when the Aeroprobe was positioned either in the trachea or the main bronchus (approximate diameter of 10 mm), the larger airway diameter reduced the impaction of the aerosol on the airway wall and led to a more even distribution in the lung. The predicted aerosol droplet size generated by this catheter is considerably larger (5–10 μm) than would normally be expected to result in deposition in the terminal bronchioles or alveolar regions. However, intratracheal aerosolization of similar size droplets to the rabbit lung, or of droplets with a mass median aerodynamic diameter of >30 μm in the mouse, has been shown to give deposition to airways of all sizes and even into the alveolar regions[17,18] suggesting that the droplet size is not so critical when generated intratracheally.

(4) We did not detect any infectious virus shedding 48 h after transfection and were, therefore, able to house treated sheep in outside pens without any specific precautions for operators and waste disposal after this time-point. Before 48 h post-dosing, animals were housed in indoor pens and all waste was autoclaved consistent with guidelines for working with genetically modified organisms.

(5) Administration of ΔF/SeV-Luc lead to gene expression 3.5 logs above untransfected controls in bronchial biopsies (unpublished data, DF/SeV-Luc: 7918 ± 2634, untransfected: 1.55 ± 0.37 RLU mg^{-1} protein $n=3$), whereas ΔF/SeV-LacZ transfection only increased β-gal by 1 log compared with control, when quantified with a chemiluminescent reporter gene assay. In cell lysates prepared from lung tissue pieces these differences were even more striking. ΔF/SeV-Luc administration increased luc expression by 5-logs (unpublished data, DF/SeV-Luc: 21079 ± 6659, untransfected: 0.07 ± 0.006 RLU mg^{-1} protein $n=3$), whereas β-gal was not reliably detectable after ΔF/SeV-LacZ transfection. For quantification of reporter gene expression in cell lysates luciferase appears to be a more reliable and sensitive reporter gene in sheep lung, consistent with results observed in murine lungs (unpublished data). This is likely due to the red colour of the tissue lysate interfering with the chemiluminescent β-gal assay used in this study.

SeV may not be the vector of choice for gene therapy for lung diseases requiring longer-term expression because expression levels after re-administration of the vector are low because of the induction of potent cellular and humoral immune responses against the virus, although the relative importance of humoral vs cellular immunity in this process is unclear. In addition to neutralising antibodies, activation of cytotoxic T-cells natural killer (NK) cells are activated through interaction of the SeV hemagluttin-neuraminidase (HN) protein with the NK cell receptor NKp46[19] having an important role. The effect of SeV on immune modulation is further highlighted in a study by Komary *et al*, which showed that SeV-transduced dendritic cells can induce persistent NK and CD4-cell-dependent anti-tumor activity.[20] We have previously shown that SeV administration evokes cellular and humoral immune responses in an animal model and that the level of neutralising antibodies (as measured by *in vitro* assays) increases after virus re-administration.[7] However, there was no correlation between neutralising antibody levels and residual gene expression ($r^2=0.046$, $P=0.16$). We have also shown that partial T-cell tolerance to SeV infection can be induced in mice after administration of immunodominant CD4 peptide eptiopes, but that a reduction in T-cells does not alter the levels of SeV-neutralising antibodies, or allow for repeat administration of the virus. Although *in vitro* quantification of neutralising antibodies is frequently undertaken, we do not believe that a single host defence factor will reliably predict efficiency of repeat administration in pre-clinical studies. Our concern about quantification of neutralising antibodies, in part, stems from recent studies showing that (a) adeno-associated virus vector can be re-administered even in the presence of high levels of circulating neutralising antibodies[21] and (b) *in vitro* neutralization assays fail to predict inhibition by antiviral antibodies *in vivo*.[22]

However, the vector remains one of the most efficient gene transfer agents for the lung. The envelope proteins F (fusion) and HN (hemagglutinin-neuraminidase) are the key factors in determining the high-transfection efficiency to airways. We have, therefore, recently pseudotyped a simian lentiviral vector with the SeV F and HN proteins (F/HN-SIV)[23] and have shown that the virus efficiently transfects mouse airway epithelium *in vivo*, as well as human air–liquid interface cultures.[24] Moreover, F/HN-SIV-mediated gene expression persists for more than 17 months after a single administration and repeated administration (3 doses) is feasible.[24] It is interesting that the HN-mediated activation of NK cells does not appear to interfere with F/HN-SIV-mediated gene expression. The F/HN-SIV vector may, therefore, be ideally suited for pulmonary gene therapy.

In conclusion, although SeV transduces the airway epithelium of mice and sheep efficiently, and retains comparatively high levels of gene expression after administration of a second dose, these levels drop further following the administration of a third dose at weekly or monthly intervals. Responses to repeat administration of SeV in natural hosts (mice) and non-natural hosts (sheep) are, therefore, similar. We suggest that SeV is not suitable for diseases that require high-level gene expression after repeat administration, but should be strongly considered for lung-related applications requiring a single administration of the gene transfer vector.

MATERIALS AND METHODS

Virus preparation

The generation and propagation of the recombinant ΔF/SeV vector carrying a luciferase (ΔF/SeV-luc) or LacZ (ΔF/SeV-LacZ) reporter gene or no reporter gene (ΔF/SeV-empty) was carried out as previously described.[25] The supernatant of LLC-MK2/F7 cells containing infectious particles was subsequently purified, concentrated and stored at −80 °C. Virus titre was determined by infecting LLC-MK2 cells and counting the number of β-gal-expressing cells after X-gal staining or by using an anti-SeV antibody and fluorescent immunohistochemistry. The titre was expressed as CIUs per ml.

Virus stability in delivery devices

PariLC plus. Two nebuliser set-ups were assessed *in vitro*. (a) Nebuliser with mouth-piece, (b) Nebuliser with attached endotracheal tube used for aerosol delivery to sheep. 5 ml of ΔF/SeV-LacZ (10^7 CIU ml^{-1}) were nebulized using a pressure of 29 psi until run dry (approximately 15 min). Aerosols were collected in a plastic tube. Polythylene endoscopic wash catheter (PEC, Olympus): 5 ml of ΔF/SeV-LacZ (10^7 CIU ml^{-1}) were delivered through the catheter and collected in a plastic tube. Trudell AeroProbe catheter (Trudell Medical International, Ontario, Canada). This is a multi-lumen catheter, with liquid injected down a central lumen and sheared into droplets at the distal tip by high-pressure air travelling down six peripheral lumens. 5 ml of ΔF/SeV-LacZ (10^7 CIU ml^{-1}) were delivered through the AeroProbe and collected in a plastic tube. To quantify virus viability, confluent LLC-MK2 cells grown in six-well plates were transfected in triplicate with appropriate virus dilutions (100 μl/plate). After 1 h cells were washed once with PBS after which 2 ml of MEM+10 foetal bovine serum and 1% penicillin/streptomycin were added. X-gal staining was performed 48 h after transfection. X-gal-positive cells were quantified in each well. Virus not exposed to nebulisers or catheters was used as a positive control and untransfected cells were used as a negative control. Virus titre (CIU ml^{-1}) was defined as X-gal positive per ml and % cell viability was calculated.

Preparation of GL67A/DNA complexes

A eukaryotic expression plasmid carrying the luciferase reporter gene cDNA (pCIKLux) under the control of the human cytomegalovirus immediate early promoter/enhancer (CMV) was used. Cationic lipid GL67A was supplied by Genzyme Corporation (Framingham, MA, USA) and complexed with DNA as previously described for intrapulmonary administration by nasal 'sniffing'.[26]

In vivo gene transfer in mice and sheep

All experiments were carried out with approval of appropriate local Ethics Committees and according to Home Office regulations.

Mice. Female Balb/C mice (6–8 weeks) were anaesthetised with metofane (Medical Developments Australia, Springvale, Australia). For intrapulmonary administration by nasal 'sniffing' a single 100 μl bolus containing either virus (10^8 CIU/mouse or GL67A/DNA complexes (80 μg plasmid DNA complexed to GL67A at a 1:4 lipid:DNA molar ratio) was slowly pipetted onto the nose and the solution was rapidly sniffed into the lungs. At indicated time-points the administration was repeated, or animals were culled and lung tissue retrieved for analysis of luciferase expression.

Sheep. Suffolk Cross ewes, 35–60 kg, were treated with anthelminthic agents before the study began and underwent a preliminary examination involving bronchoscopic visualization and bronchoalveolar lavage of segment right apical under gaseous anaesthesia 1–3 weeks before treatment to confirm absence of pre-existing pulmonary disease. Anaesthetized sheep were maintained in a whole body, negative pressure respirator as described previously.[8] Virus was delivered by means of a bronchoscope, either by (a) instillation to single lung segments via the PEC, (b) by AeroProbe catheter directed at specific locations, or (c) by AeroProbe catheter directed at the whole lung. For instillation to individual lung segments the bronchoscope was wedged within that segment and either 0.67×10^9 CIU ml^{-1} of ΔF/SeV-LacZ (segment right caudal diaphragmatic) or empty vector (ΔF/SeV-empty (segment left caudal diaphragmatic) instilled in 5 ml PBS. For AeroProbe delivery to individual segments the tip of the AeroProbe was positioned in the lumen at the entrance to the segment, but not wedged, as 50-psi air pressure is required to generate the aerosol. 2×10^9 CIU ml^{-1} were delivered in 5 ml PBS.

For delivery to the whole lung the AeroProbe was positioned centrally in the trachea, immediately distal to the end of the endotracheal tube and 1.6×10^9 CIU ml^{-1} delivered in 24 ml PBS. For duration of expression and repeat dose studies a 7 ml dose of virus containing 5×10^9 or 1×10^{10} CIU was delivered to each side of the lungs, respectively. With the Aeroprobe catheter positioned far back in the right and left main bronchi the aerosol exposure was focussed in the caudal and ventral diaphragmatic lung segments.

At the indicated time points the temperature of the animal was recorded and blood samples collected for serum and haematology. The sheep were either killed for analysis, anaesthetized as before for repeat administration of virus or anaesthetized and maintained on a positive pressure ventilation system (Harvard Apparatus Model 708) for the collection of bronchial biopsies and bronchial brushings with biopsy forceps (Crocodile Biopsy Forceps, Olympus FB-15K-1, Olympus Keymed, Essex, UK) or cytology brushes (Olympus BC-202D-2010), respectively. Bronchial brushings were collected into PBS and biopsies frozen for luciferase assays. Following euthanasia by lethal injection and exsanguination, the lungs were removed for tissue harvesting. The pulmonary circulation was flushed through the pulmonary artery with 2–3 l of PBS before sampling. bronchoalveolar lavage fluid was collected as for pre-treatment sampling but from segment right caudal diaphragmatic. Lung tissues collected post mortem were fixed by perfusion with 2% neutral buffered formalin, 0.2% glutaraldehyde, 2 mm $MgCl_2$ and 5 mM EGTA pH 8 in 0.1 M phosphate buffer (pH 7.3) and selected segments dissected out for X-gal staining. Alternatively, individual segments were cut transversely into approximately 1-cm thick slices representing upper, middle and lower regions. Individual airways (>2 mm diameter) were dissected from the upper region (Airway Upper). Parenchymal samples (containing airways too small to dissect, <2 mm diameter) were derived from the upper (parenchyma upper), middle (M) and lower (L) regions. Finely chopped samples were snap frozen for luciferase assays.

Reporter gene expression

Luciferase assay. Mouse right lungs were placed in 300 μl 1×RLB buffer (Promega, Southampton, UK) and homogenized, followed by three freeze–thaw cycles (−80 °C for 30 min, thawed at room temperature) and centrifugation (10 min at 13 000g_{av}). Sheep lung tissue (300 mg) or whole-bronchial biopsies were transferred to FastRNA ProGreen matrix tubes (MP Biomedicals, Solon, OH, USA) in 600 μl reporter lysis buffer (RLB; Promega Corp., Madison, WI, USA) and homogenized in a FastPrep Instrument (MP Biomedicals) for 40 s at speed setting 6. Lysates were centrifuged at 13 000 g_{av}/10 min/4 °C. Supernatant was frozen on dry ice and stored at −80 °C. All supernatants were removed and frozen at −80 °C for luciferase quantification. Luciferase activity was measured in the supernatant using a standard luciferase assay kit (Promega) and the TD-20e luminometer (Turner BioSystems, Sunnyvale, CA, USA) or the Anthos Lucy1 luminometer (Labtech International, East Sussex, UK). *β-gal assay in tissue lysate*: sheep lung tissue was homogenized (Ultra-Turrax homogeniser Science Lab Houston, TX, USA) in 1×RLB buffer by weight (1 ml RLB to 1 g tissue and bronchial biopsies were homogenized by hand using scissors in 120 μl 1×RLB buffer. Supernatants from the homogenates were collected after three freeze–thaw cycles (−80 °C for 30 min, thawed at room temperature) and centrifugation (10 min at 13 000g_{av}). Samples were stored −80 °C β-gal expression was quantified using the Clontech Detection Kit II (BD Biosciences Clontech, Franklin Lakes, NJ, USA). Light emission was measured by the TD-20e luminometer as described above. *Protein assay*: total protein per sample was determined using the BioRad protein assay kit (BioRad laboratories, Hercules, CA, USA) and luciferase or β-gal activity was expressed as arbitrary relative light units per mg total protein. *X-gal staining*: lung tissue was stained as described previously.[27] Briefly, fixed sheep tissue was washed three times in detergent (20 min each) and stained in X-gal for up to 48 h at 30 °C. Samples were then washed (2×20 min in PBS containing 2 mM $MgCl_2$) and then processed into wax for sectioning.

Virus shedding

Sheep (n=5) were transfected with ΔF/SeV-LacZ using whole-lung Trudell AeroProbe administration (10^{10} CIU in 24 ml PBS). At 48 h after delivery bronchoalveolar lavage, oral and nasal swabs in PBS and lung tissue samples were collected from two sheep at necropsy. For the remaining three sheep nasal and oral swabs, broncho-alveolar lavage fluid and serum were collected from sheep that were kept alive as part of the 28-day duration study. These samples were analyzed for viable virus particles. Lung tissue was homogenized in PBS and all samples were filtered (0.45 μm) to allow removal of bacteria and fungi. Moreover, filters were first treated with 1 ml of ΔF/SeV-GFP (10^7 CIU ml^{-1}) to minimise ΔF/SeV-LacZ absorption to the filter during this process. Viable virus particles were then quantified by incubating the samples with LLC-MK2 cells as described above.

To determine assay sensitivity, PBS was 'spiked' with known amounts of virus (7 to 7000 CIU ml^{-1}), filtered through a 0.45 μm filter and plated onto LLCMK cells for X-gal staining. The assay was able to detect 1:250 viable virus particles.

Statistical analysis

Statistical analyses were performed by ANOVA or Kruskal–Wallis followed by *post-hoc* analysis appropriate for parametric and non-parametric data. The null hypothesis was rejected at $P<0.05$.

CONFLICT OF INTEREST

MH and TS are members of the corporate management of DNAVEC Corporation. The remaining authors declare no conflict of interest.

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