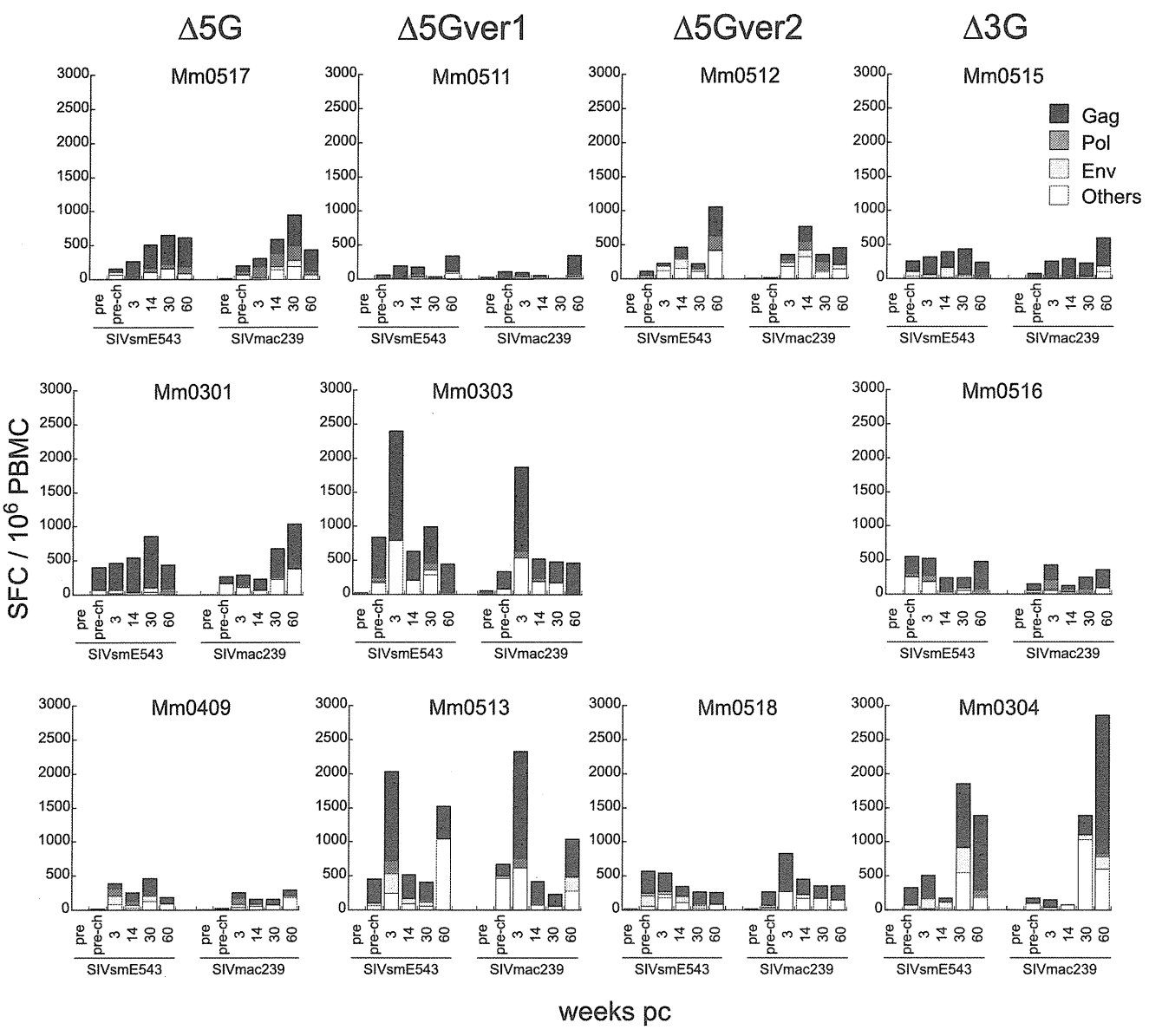

Figure 6. SIV specific cellular response in the vaccine recipients. SIV specific T cells were stimulated with overlapping peptides encompassing the viral proteins (Gag, Pol, Env, and Others: Vif, Vpr, Vpx, Tat, Rev, and Nef) of SIVmac239 and SIVsmE543-3 and the number of spot forming cells (SFC) per 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

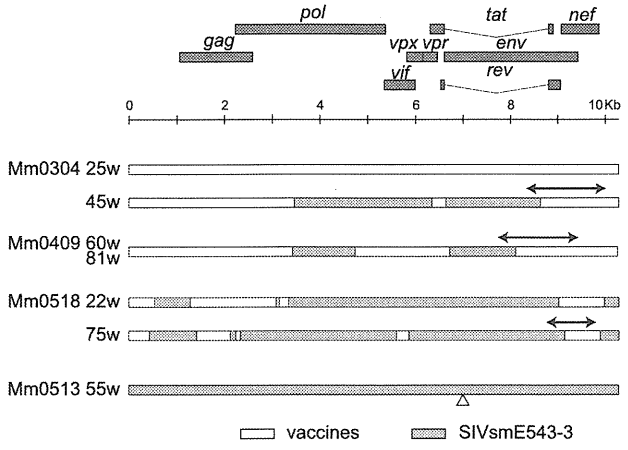

Figure 7. Recombination between vaccine and challenge virus. Nucleotide sequences of SIV fragments amplified by sets of nested PCR using primers based on SIVsmE543-3 or SIVmac239 to cover the entire SIV genome except the 5′ and 3′ terminal sequences (~100 bp). Representative sequences from integrated 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

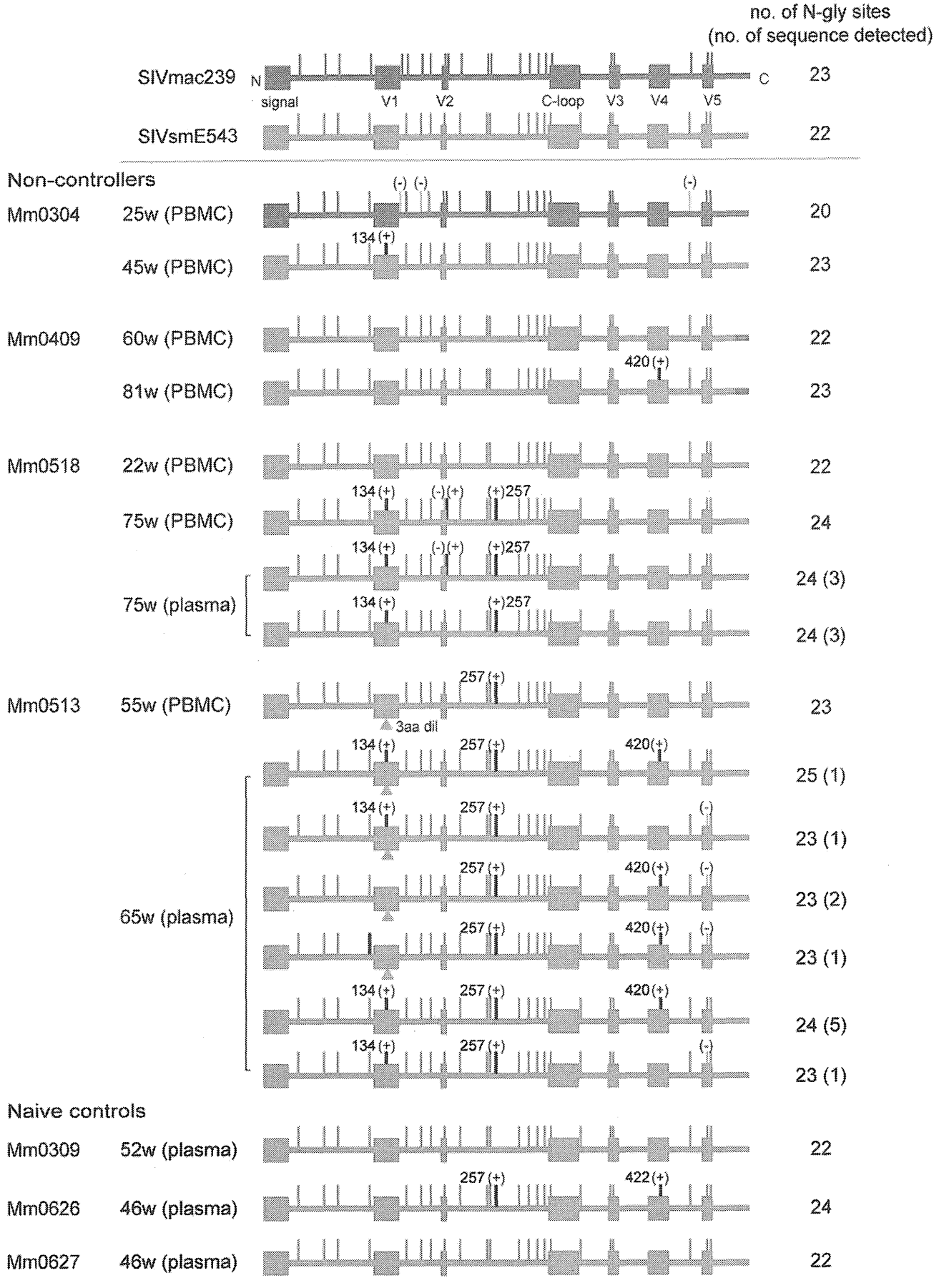
no. of N-gly sites
(no. of sequence detected)
SIVmac239
N
C
signal
V1
V2
C-loop
V3
V4
V5
23
SIVsmE543
22
Non-controllers
Mm0304
25w (PBMC)
(-) (-)
(-)
20
45w (PBMC)
134 (+)
23
Mm0409
60w (PBMC)
22
81w (PBMC)
420 (+)
23
Mm0518
22w (PBMC)
22
75w (PBMC)
134 (+)
(-)(+)
(+)257
24
75w (plasma)
24 (3)
24 (3)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

References

1. Gaschen B, Taylor J, Yusim K, Foley B, Gao F, et al. (2002) Diversity considerations in HIV-1 vaccine selection. Science 296: 2354–2360.
2. Ohgimoto S, Shioda T, Mori K, Nakayama EE, Hu H, et al. (1998) Location-specific, unequal contribution of the N glycans in simian immunodeficiency virus gp120 to viral infectivity and removal of multiple glycans without disturbing infectivity. J Virol 72: 8365–8370.
3. Mori K, Yasutomi Y, Ohgimoto S, Nakasone T, Takamura S, et al. (2001) Quintuple deglycosylation mutant of simian immunodeficiency virus SIV-mac239 in rhesus macaques: robust primary replication, tightly contained chronic infection, and elicitation of potent immunity against the parental wild-type strain. J Virol 75: 4023–4028.
4. Hirsch V, Adger-Johnson D, Campbell B, Goldstein S, Brown C, et al. (1997) A molecularly cloned, pathogenic, neutralization-resistant simian immunodeficiency virus, SIVsmE543-3. J Virol 71: 1608–1620.
5. Loffredo JT, Bean AT, Beal DR, Leon EJ, May GE, et al. (2008) Patterns of CD8+ immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication. J Virol 82: 1723–1738.
6. Yant LJ, Friedrich TC, Johnson RC, May GE, Maness NJ, et al. (2006) The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J Virol 80: 5074–5077.
7. O'Connor DH, Mothe BR, Weinfurter JT, Fuenger S, Rehrauer WM, et al. (2003) Major histocompatibility complex class I alleles associated with slow simian immunodeficiency virus disease progression bind epitopes recognized by dominant acute-phase cytotoxic-T-lymphocyte responses. J Virol 77: 9029–9040.
8. Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, et al. (2004) Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J Exp Med 199: 1709–1718.
9. Goulder PJ, Watkins DI (2008) Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat Rev Immunol 8: 619–630.
10. Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, et al. (2000) HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc Natl Acad Sci U S A 97: 2709–2714.
11. Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, et al. (2007) CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 13: 46–53.
12. Hendel H, Caillat-Zucman S, Lebuanec H, Carrington M, O'Brien S, et al. (1999) New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS. J Immunol 162: 6942–6946.
13. Apetrei C, Kaur A, Lerche NW, Metzger M, Pandrea I, et al. (2005) Molecular epidemiology of simian immunodeficiency virus SIVsm in U.S. primate centers unravels the origin of SIVmac and SIVstm. J Virol 79: 8991–9005.
14. Johnson WE, Sanford H, Schwall L, Burton DR, Parren PW, et al. (2003) Assorted mutations in the envelope gene of simian immunodeficiency virus lead to loss of neutralization resistance against antibodies representing a broad spectrum of specificities. J Virol 77: 9993–10003.
15. Sugimoto C, Nakayama EE, Shioda T, Villinger F, Ansari AA, et al. (2008) Impact of glycosylation on antigenicity of simian immunodeficiency virus SIV239: induction of rapid V1/V2-specific non-neutralizing antibody and delayed neutralizing antibody following infection with an attenuated deglycosylated mutant. J Gen Virol 89: 554–566.
16. Mori K, Sugimoto C, Ohgimoto S, Nakayama EE, Shioda T, et al. (2005) Influence of glycosylation on the efficacy of an Env-based vaccine against simian immunodeficiency virus SIVmac239 in a macaque AIDS model. J Virol 79: 10386–10396.
17. Sugimoto C, Tadakuma K, Otani I, Moritoyo T, Akari H, et al. (2003) nef gene is required for robust productive infection by simian immunodeficiency virus of T-cell-rich paracortex in lymph nodes. J Virol 77: 4169–4180.
18. Wei X, Decker JM, Wang S, Hui H, Kappes JC, et al. (2003) Antibody neutralization and escape by HIV-1. Nature 422: 307–312.
19. Reitter JN, Means RE, Desrosiers RC (1998) A role for carbohydrates in immune evasion in AIDS. Nat Med 4: 679–684.
20. Chackerian B, Rudensey LM, Overbaugh J (1997) Specific N-linked and O-linked glycosylation modifications in the envelope V1 domain of simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies. J Virol 71: 7719–7727.
21. Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, et al. (2002) Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. J Virol 76: 2298–2305.
22. Tsukamoto T, Dohki S, Ueno T, Kawada M, Takeda A, et al. (2008) Determination of a major histocompatibility complex class I restricting simian immunodeficiency virus Gag241–249 epitope. Aids 22: 993–994.
23. Reynolds MR, Weiler AM, Weisgrau KL, Piaskowski SM, Furlott JR, et al. (2008) Macaques vaccinated with live-attenuated SIV control replication of heterologous virus. J Exp Med 205: 2537–2550.
24. Wyand MS, Manson K, Montefiori DC, Lifson JD, Johnson RP, et al. (1999) Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. J Virol 73: 8356–8363.
25. Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, et al. (1990) Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science 248: 1109–1112.
26. Casimiro DR, Wang F, Schleif WA, Liang X, Zhang ZQ, et al. (2005) Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with dna and recombinant adenoviral vaccine vectors expressing Gag. J Virol 79: 15547–15555.
27. Reimann KA, Parker RA, Seaman MS, Beaudry K, Beddall M, et al. (2005) Pathogenicity of simian-human immunodeficiency virus SHIV-89.6P and SIVmac is attenuated in cynomolgus macaques and associated with early T-lymphocyte responses. J Virol 79: 8878–8885.
28. Kim EY, Busch M, Abel K, Fritts L, Bustamante P, et al. (2005) Retroviral recombination in vivo: viral replication patterns and genetic structure of simian immunodeficiency virus (SIV) populations in rhesus macaques after simultaneous or sequential intravaginal inoculation with SIVmac239Deltavpx/Deltavpr and SIVmac239Deltanef. J Virol 79: 4886–4895.
29. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.
30. Regier DA, Desrosiers RC (1990) The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res Hum Retroviruses 6: 1221–1231.
31. Means RE, Greenough T, Desrosiers RC (1997) Neutralization sensitivity of cell culture-passaged simian immunodeficiency virus. J Virol 71: 7895–7902.

Acknowledgments

This study was conducted through the Cooperative Research Program in Tsukuba Primate Research Center, National Institute of Biomedical Innovation. We would like to acknowledge all the animal care and veterinary staff at the Tsukuba Primate Research Center. We would like to thank Yutaka Takebe, Atsushi Komano, and Yasuhiro Yasutomi for helpful discussion of the results in this study.

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.

Gene Therapy (2011) 18, 182–188

www.nature.com/gt

ORIGINAL ARTICLE

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

U Griesenbach[1,4,5], G McLachlan[2,4,5], T Owaki[3], L Somerton[1,4], T Shu[3], A Baker[2,4], P Tennant[2,4], C Gordon[2,4], C Vrettou[2,4], E Baker[2,4], DDS Collie[2,4], M Hasegawa[3] and EWFW Alton[1,4]

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.
Gene Therapy (2011) **18,** 182–188; doi:10.1038/gt.2010.131; published online 21 October 2010

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

[1]Department of Gene Therapy, Imperial College London, National Heart and Lung Institute, London, UK; [2]The Wellcome Trust Centre for Research in Comparative Respiratory Medicine, The Roslin Institute & R(D)SVS, University of Edinburgh, UK; [3]DNAVEC Corporation, Tsukuba, Japan and [4]UK CF Gene Therapy Consortium, UK
Correspondence: Dr U Griesenbach, Department of Gene Therapy, Imperial College at the National Heart and Lung Institute, Manresa Road, London SW3 6LR, UK.
E-mail: u.griesenbach@imperial.ac.uk
[5]These authors contributed equally to this work.
Received 19 May 2010; revised 27 July 2010; accepted 9 August 2010; published online 21 October 2010

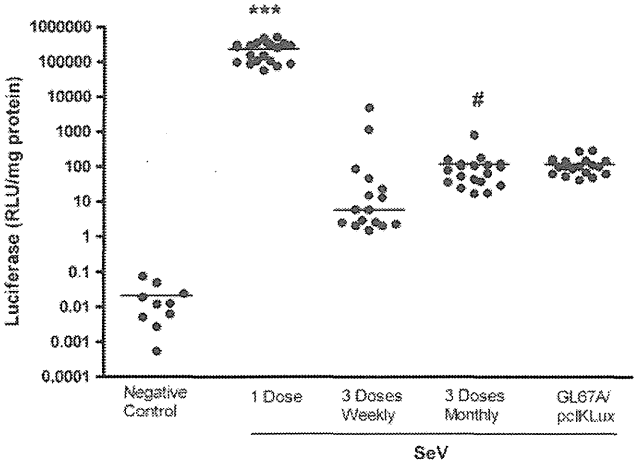

Figure 1 Repeat administration of ΔF/SeV in mouse lung. Murine lungs were transfected with three doses (10^8 CIU/mouse/dose) of transmission incompetent ΔF/SeV at either weekly or monthly intervals by nasal sniffing. To circumvent generation of anti-reporter gene immune responses a vector without a reporter 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

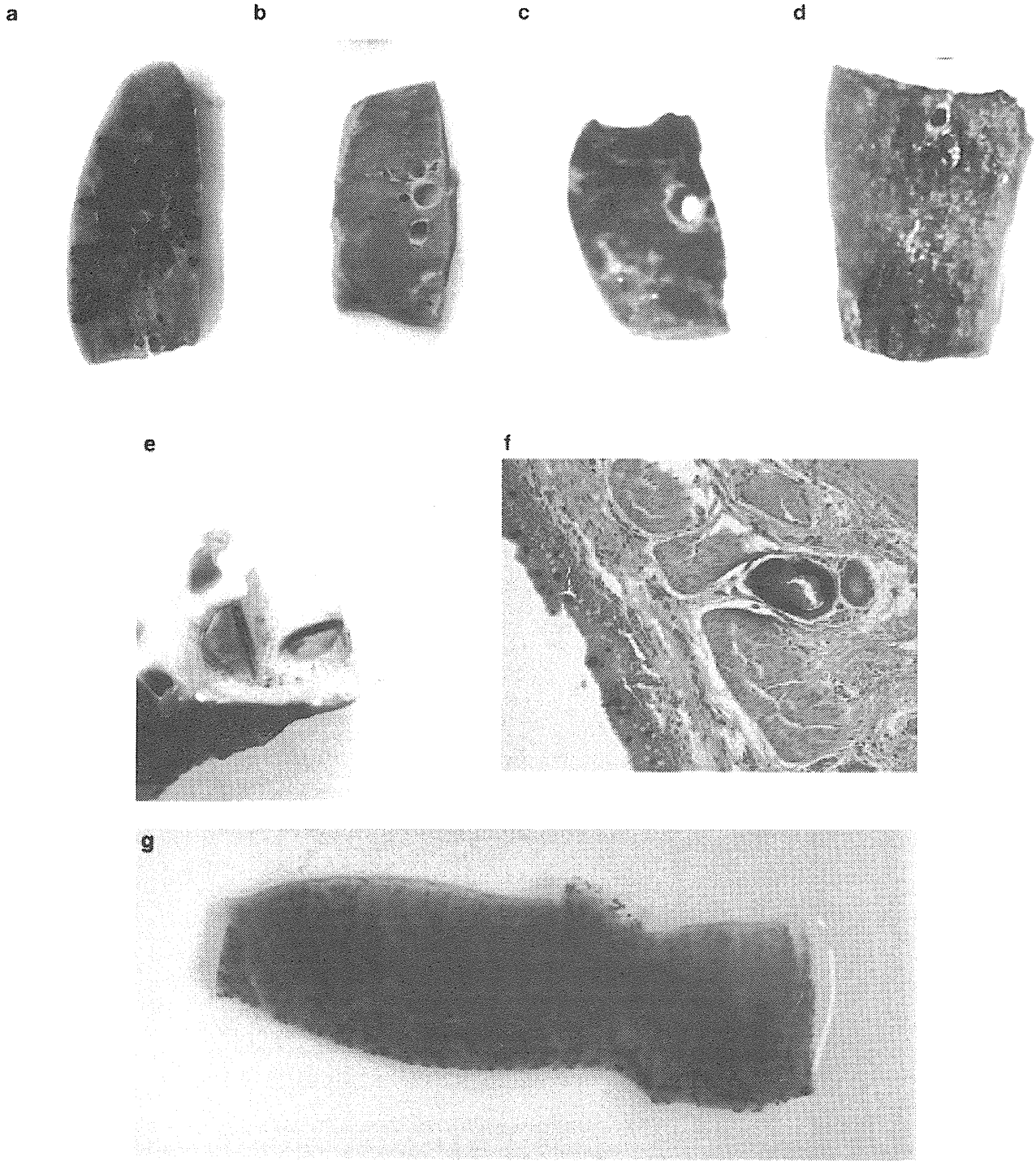

Figure 2 β-gal expression in sheep lung 2 days after transfection with ΔF/SeV-LacZ delivered with either a polyethylene catheter (PEC) or the Trudell AeroProbe. ΔF/SeV-LacZ was administered to a single segment of the sheep lung with a PEC (3.4×10^9 CIU/sheep in 5 ml, n=3 sheep). High level, but patchy, 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

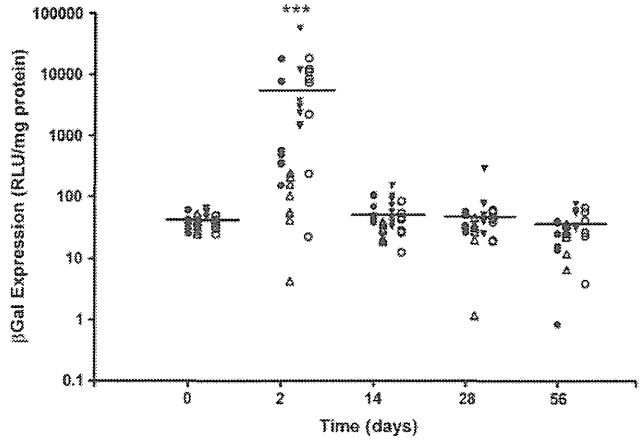

Figure 3 Duration of gene expression in sheep lung. Sheep were transfected with ΔF/SeV-LacZ (10^{10} CIU in 24 ml per sheep, n=4 sheep) and biopsies were collected 2, 14, 28 and 52 days after transfection (n=8/sheep/time point) to follow gene expression over time. Each symbol represents one biopsy. Horizontal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

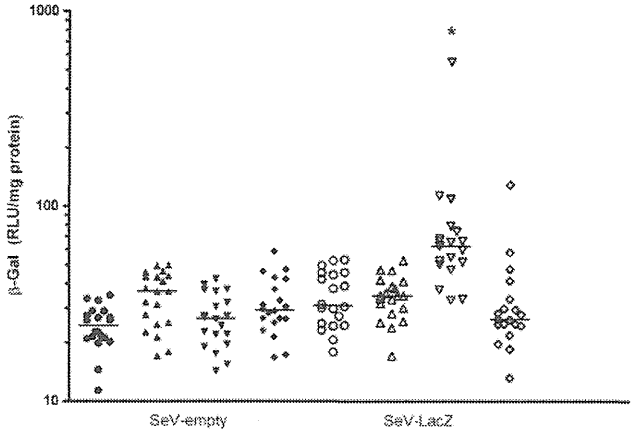

Figure 4 Repeat administration (3 doses monthly) of ΔF/SeV-LacZ in sheep. Sheep lung was transfected with three doses (10^{10} CIU/sheep/dose) of ΔF/SeV at monthly intervals. To circumvent generation of anti-reporter gene immune responses ΔF/SeV-empty was administered for the first two doses. ΔF/SeV-LacZ 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.

ACKNOWLEDGEMENTS

We thank Dr M Inoue (DNAVEC, Corporation) for help with preparing the manuscript. This work was in part funded by the Cystic Fibrosis Trust and a CF Trust Senior Fellowship (UG). The work was supported by the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London.

1 Ferrari S, Griesenbach U, Shiraki-Iida T, Shu T, Hironaka T, Hou X *et al.* A defective nontransmissible recombinant Sendai virus mediates efficient gene transfer to airway epithelium *in vivo*. *Gene Ther* 2004; **11**: 1659–1664.
2 Yonemitsu Y, Kitson C, Ferrari S, Farley R, Griesenbach U, Judd D *et al.* Efficient gene transfer to airway epithelium using recombinant Sendai virus. *Nat Biotechnol* 2000; **18**: 970–973.
3 Ferrari S, Griesenbach U, Iida A, Farley R, Wright AM, Zhu J *et al.* Sendai virus-mediated CFTR gene transfer to the airway epithelium. *Gene Ther* 2007; **14**: 1371–1379.
4 Li HO, Zhu YF, Asakawa M, Kuma H, Hirata T, Ueda Y *et al.* A cytoplasmic RNA vector derived from nontransmissible sendai virus with efficient gene transfer and expression. *J Virol* 2000; **74**: 6564–6569.
5 Halbert CL, Rutledge EA, Allen JM, Russell DW, Miller AD. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. *J Virol* 2000; **74**: 1524–1532.
6 Sumner-Jones SG, Gill DR, Hyde SC. Lack of repeat transduction by recombinant adeno-associated virus type 5/5 vectors in the mouse airway. *J Virol* 2007; **81**: 12360–12367.
7 Griesenbach U, Boyton RJ, Somerton L, Garcia SE, Ferrari S, Owaki T *et al.* Effect of tolerance induction to immunodominant T-cell epitopes of Sendai virus on gene expression following repeat administration to lung. *Gene Ther* 2006; **13**: 449–456.
8 McLachlan G, Baker A, Tennant P, Gordon C, Vrettou C, Renwick L *et al.* Optimizing aerosol gene delivery and expression in the ovine lung. *Mol Ther* 2007; **15**: 348–354.
9 Auricchio A, O'Connor E, Weiner D, Gao GP, Hildinger M, Wang L *et al.* Noninvasive gene transfer to the lung for systemic delivery of therapeutic proteins. *J Clin Invest* 2002; **110**: 499–504.
10 Fischer AC, Beck SE, Smith CI, Laube BL, Askin FB, Guggino SE *et al.* Successful transgene expression with serial doses of aerosolized rAAV2 vectors in rhesus macaques. *Mol Ther* 2003; **8**: 918–926.
11 Koehler DR, Martin B, Corey M, Palmer D, Ng P, Tanswell AK *et al.* Readministration of helper-dependent adenovirus to mouse lung. *Gene Ther* 2006; **13**: 773–780.
12 Harvey BG, Leopold PL, Hackett NR, Grasso TM, Williams PM, Tucker AL *et al.* Airway epithelial CFTR mRNA expression in cystic fibrosis patients after repetitive administration of a recombinant adenovirus. *J Clin Invest* 1999; **104**: 1245–1255.
13 Eastman SJ, Tousignant JD, Lukason MJ, Murray H, Siegel CS, Constantino P *et al.* Optimization of formulations and conditions for the aerosol delivery of functional cationic lipid:DNA complexes. *Hum Gene Ther* 1997; **8**: 313–322.
14 Leung K, Louca E, Munson K, Dutzar B, Anklesaria P, Coates AL. Calculating expected lung deposition of aerosolized administration of AAV vector in human clinical studies. *J Gene Med* 2007; **9**: 10–21.
15 Alton EW, Stern M, Farley R, Jaffe A, Chadwick SL, Phillips J *et al.* Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. *Lancet* 1999; **353**: 947–954.
16 Moss RB, Rodman D, Spencer LT, Aitken ML, Zeitlin PL, Waltz D *et al.* Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest* 2004; **125**: 509–521.
17 Koehler DR, Frndova H, Leung K, Louca E, Palmer D, Ng P *et al.* Aerosol delivery of an enhanced helper-dependent adenovirus formulation to rabbit lung using an intra-tracheal catheter. *J Gene Med* 2005; **7**: 1409–1420.
18 Koping-Hoggard M, Issa MM, Kohler T, Tronde A, Varum KM, Artursson P. A miniaturized nebulization catheter for improved gene delivery to the mouse lung. *J Gene Med* 2005; **7**: 1215–1222.
19 Arnon TI, Achdout H, Lieberman N, Gazit R, Gonen-Gross T, Katz G *et al.* The mechanisms controlling the recognition of tumor- and virus-infected cells by NKp46. *Blood* 2004; **103**: 664–672.
20 Komaru A, Ueda Y, Furuya A, Tanaka S, Yoshida K, Kato T *et al.* Sustained and NK/CD4+ T cell-dependent efficient prevention of lung metastasis induced by dendritic cells harboring recombinant Sendai virus. *J Immunol* 2009; **183**: 4211–4219.
21 Limberis MP, Wilson JM. Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered. *Proc Natl Acad Sci USA* 2006; **103**: 12993–12998.
22 Pichla-Gollon SL, Lin SW, Hensley SE, Lasaro MO, Herkenhoff-Haut L, Drinker M *et al.* Effect of preexisting immunity on an adenovirus vaccine vector: *in vitro* neutralization assays fail to predict inhibition by antiviral antibody *in vivo*. *J Virol* 2009; **83**: 5567–5573.
23 Kobayashi M, Iida A, Ueda Y, Hasegawa M. Pseudotyped lentivirus vectors derived from simian immunodeficiency virus SIVagm with envelope glycoproteins from paramyxovirus. *J Virol* 2003; **77**: 2607–2614.
24 Mitomo K, Griesenbach U, Inoue M, Somerton L, Meng C, Akiba E *et al.* Toward Gene Therapy for Cystic Fibrosis Using a Lentivirus Pseudotyped With Sendai Virus Envelopes. *Mol Ther* 2010; **18**: 1173–1182.
25 Hirata T, Iida A, Shiraki-Iida T, Kitazato K, Kato A, Nagai Y *et al.* An improved method for recovery of F-defective Sendai virus expressing foreign genes from cloned cDNA. *J Virol Methods* 2002; **104**: 125–133.
26 Lee ER, Marshall J, Siegel CS, Jiang C, Yew NS, Nichols MR *et al.* Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum Gene Ther* 1996; **7**: 1701–1717.
27 McLachlan G, Davidson DJ, Stevenson BJ, Dickinson P, Vidson-Smith H, Dorin JR *et al.* Evaluation *in vitro* and *in vivo* of cationic liposome-expression construct complexes for cystic fibrosis gene therapy. *Gene Therapy* 1995; **2**: 614–622.

Biochemical and Biophysical Research Communications 408 (2011) 615–619

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

Yusuke Takahara [a,b], Saori Matsuoka [b], Tetsuya Kuwano [a], Tetsuo Tsukamoto [a], Hiroyuki Yamamoto [b], Hiroshi Ishii [a,b], Tadashi Nakasone [b], Akiko Takeda [b], Makoto Inoue [c], Akihiro Iida [c], Hiroto Hara [c], Tsugumine Shu [c], Mamoru Hasegawa [c], Hiromi Sakawaki [d], Mariko Horiike [d], Tomoyuki Miura [d], Tatsuhiko Igarashi [d], Taeko K. Naruse [e], Akinori Kimura [e], Tetsuro Matano [a,b,*]

[a] Division for AIDS Vaccine Development, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[b] AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[c] DNAVEC Corporation, 6 Ohkubo, Tsukuba, Ibaraki 300-2611, Japan
[d] Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
[e] Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

ARTICLE INFO

Article history:
Received 12 April 2011
Available online 21 April 2011

Keywords:
AIDS vaccine
HIV
SIV
CTL
Immunodominance

ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

* Corresponding author at: AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Fax: +81 3 5285 1165.
E-mail address: tmatano@nih.go.jp (T. Matano).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.04.071

$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

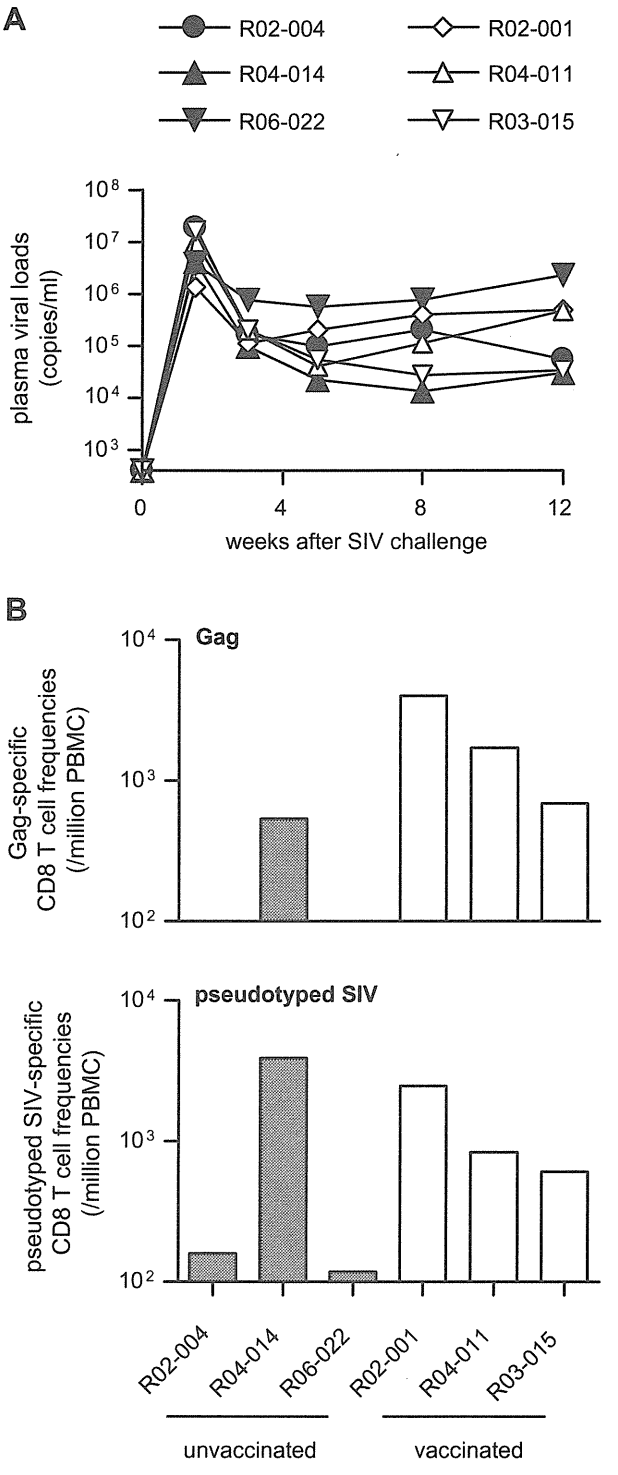

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

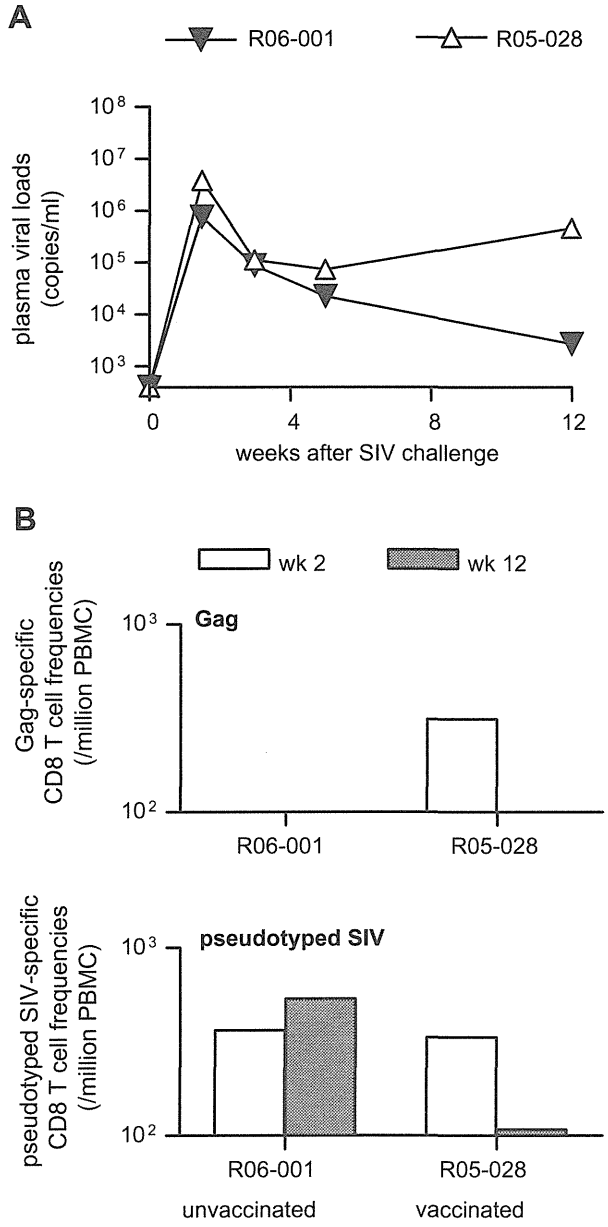

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06–001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

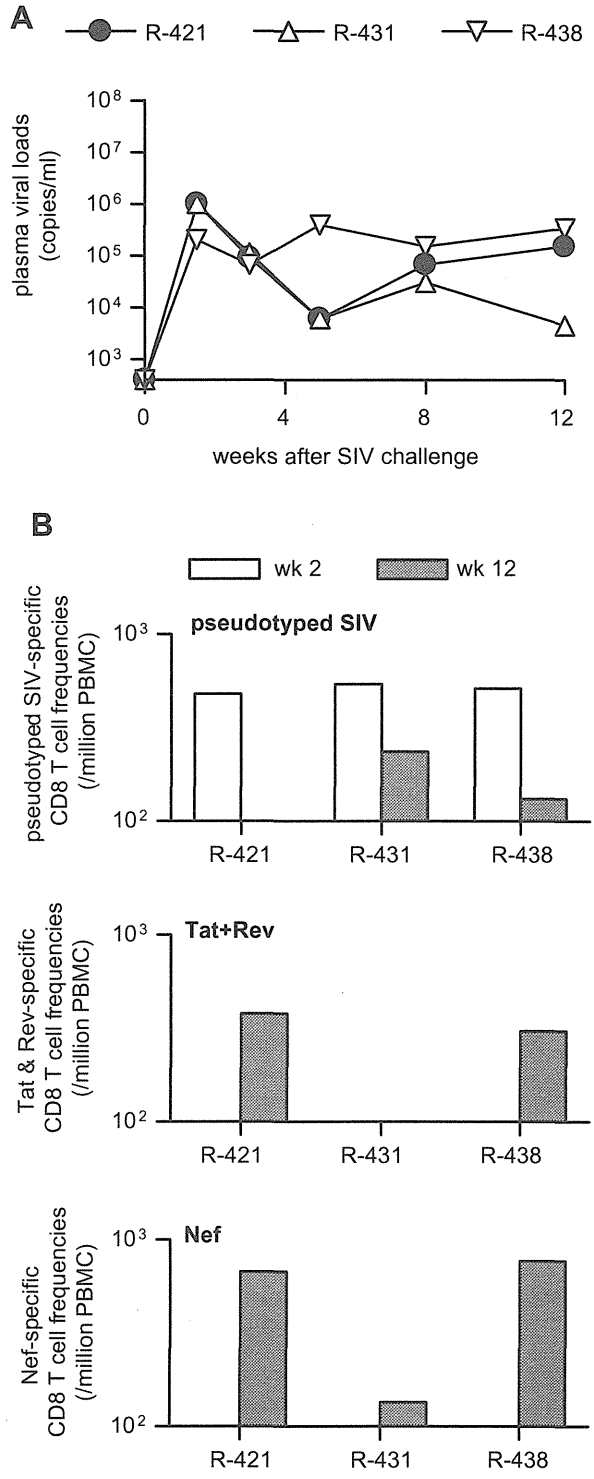

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific $CD8^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R- 421					++											
R- 431					+											
R- 438	++		+							++						
wk 12																
R- 421		++			++				+		+	+	+			++
R- 431					+		+			++						
R- 438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral

exposure [21,22]. These results imply a significant influence of prophylactic vaccination on the immunodominance pattern of CTL responses post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

Acknowledgments

This work was supported by Grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, Grants-in-aid from the Ministry of Health, Labor, and Welfare, and a Grant from Takeda Science Foundation in Japan.

References

[1] R.A. Koup, J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, D.D. Ho, Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome, J. Virol. 68 (1994) 4650–4655.
[2] P. Borrow, H. Lewicki, B.H. Hahn, G.M. Shaw, M.B. Oldstone, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection, J. Virol. 68 (1994) 6103–6110.
[3] T. Matano, R. Shibata, C. Siemon, M. Connors, H.C. Lane, M.A. Martin, Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques, J. Virol. 72 (1998) 164–169.
[4] X. Jin, D.E. Bauer, S.E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, J. Mittler, L. Weinberger, L.G. Kostrikis, L. Zhang, A.S. Perelson, D.D. Ho, Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques, J. Exp. Med. 189 (1999) 991–998.
[5] P.J. Goulder, D.I. Watkins, HIV and SIV CTL escape: implications for vaccine design, Nat. Rev. Immunol. 4 (2004) 630–640.
[6] T. Matano, M. Kano, H. Nakamura, A. Takeda, Y. Nagai, Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen, J. Virol. 75 (2001) 11891–11896.
[7] T. Matano, M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D.H. O'Connor, D.I. Watkins, Y. Nagai, Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial, J. Exp. Med. 199 (2004) 1709–1718.
[8] Y. Takahashi-Tanaka, M. Yasunami, T. Naruse, K. Hinohara, T. Matano, K. Mori, M. Miysazawa, M. Honda, Y. Yasutomi, Y. Nagai, A. Kimura, Reference strand-mediated conformation analysis (RSCA)-based typing of multiple alleles in the rhesus macaque MHC class I Mamu-A and Mamu-B loci, Electrophoresis 28 (2007) 918–924.
[9] M. Kawada, T. Tsukamoto, H. Yamamoto, N. Iwamoto, K. Kurihara, A. Takeda, C. Moriya, H. Takeuchi, H. Akari, T. Matano, Gag-specific cytotoxic T lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial, J. Virol. 82 (2008) 10199–10206.
[10] T. Tsukamoto, A. Takeda, T. Yamamoto, H. Yamamoto, M. Kawada, T. Matano, Impact of cytotoxic-T-lymphocyte memory induction without virus-specific CD4+ T-Cell help on control of a simian immunodeficiency virus challenge in rhesus macaques, J. Virol. 83 (2009) 9339–9346.
[11] H. Yamamoto, M. Kawada, A. Takeda, H. Igarashi, T. Matano, Post-infection immunodeficiency virus control by neutralizing antibodies, PLoS ONE 2 (2007) e540.
[12] R. Shibata, F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, M.A. Martin, Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing, J. Infect. Dis. 176 (1997) 362–373.
[13] H.O. Li, Y.F. Zhu, M. Asakawa, H. Kuma, T. Hirata, Y. Ueda, Y.S. Lee, M. Fukumura, A. Iida, A. Kato, Y. Nagai, M. Hasegawa, A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression, J. Virol. 74 (2000) 6564–6569.
[14] A. Takeda, H. Igarashi, H. Nakamura, M. Kano, A. Iida, T. Hirata, M. Hasegawa, Y. Nagai, T. Matano, Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model, J. Virol. 77 (2003) 9710–9715.
[15] H.W. Kestler 3rd, D.J. Ringler, K. Mori, D.L. Panicali, P.K. Sehgal, M.D. Daniel, R.C. Desrosiers, Importance of the nef gene for maintenance of high virus loads and for development of AIDS, Cell 65 (1991) 651–662.
[16] T. Matano, M. Kano, T. Odawara, H. Nakamura, A. Takeda, K. Mori, T. Sato, Y. Nagai, Induction of protective immunity against pathogenic simian immunodeficiency virus by a foreign receptor-dependent replication of an engineered avirulent virus, Vaccine 18 (2000) 3310–3318.
[17] L.M. Albritton, L. Tweng, D. Scadden, J.M. Cunningham, A putative murine retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection, Cell 57 (1989) 659–666.
[18] M. Kawada, T. Tsukamoto, H. Yamamoto, A. Takeda, H. Igarashi, D.I. Watkins, T. Matano, Long-term control of simian immunodeficiency virus replication with central memory CD4+ T-cell preservation after nonsterile protection by a cytotoxic T-lymphocyte-based vaccine, J. Virol. 81 (2007) 5202–5211.
[19] N. Iwamoto, T. Tsukamoto, M. Kawada, A. Takeda, H. Yamamoto, H. Takeuchi, T. Matano, Broadening of CD8+ cell responses in vaccine-based simian immunodeficiency virus controllers, AIDS 24 (2010) 2777–2787.
[20] S. Tenzer, E. Wee, A. Burgevin, G. Stewart-Jones, L. Friis, K. Lamberth, C.H. Chang, M. Harndahl, M. Weimershaus, J. Gerstoft, N. Akkad, P. Klenerman, L. Fugger, E.Y. Jones, A.J. McMichael, S. Buus, H. Schild, P. van Endert, A.K. Iversen, Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance, Nat. Immunol. 10 (2009) 636–646.
[21] P.J.R. Goulder, D.I. Watkins, Impact of MHC class I diversity on immune control of immunodeficiency virus replication, Nat. Rev. Immunol. 8 (2008) 619–630.
[22] H. Streeck, J.S. Jolin, Y. Qi, B. Yassine-Diab, R.C. Johnson, D.S. Kwon, M.M. Addo, C. Brumme, J.P. Routy, S. Little, H.K. Jessen, A.D. Kelleher, F.M. Hecht, R.P. Sekaly, E.S. Rosenberg, B.D. Walker, M. Carrington, M. Altfeld, Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells, J. Virol. 83 (2009) 7641–7648.
[23] L. Alexander, L. Denekamp, S. Czajak, R.C. Desrosiers, Suboptimal nucleotides in the infectious, pathogenic simian immunodeficiency virus clone SIVmac239, J. Virol. 75 (2001) 4019–4022.

Microbiol Immunol 2011; **55**: 768–773
doi:10.1111/j.1348-0421.2011.00384.x

ORIGINAL ARTICLE

Major histocompatibility complex class I-restricted cytotoxic T lymphocyte responses during primary simian immunodeficiency virus infection in Burmese rhesus macaques

Midori Nakamura[1,2], Yusuke Takahara[1,2], Hiroshi Ishii[1,2], Hiromi Sakawaki[3], Mariko Horiike[3], Tomoyuki Miura[3], Tatsuhiko Igarashi[3], Taeko K. Naruse[4], Akinori Kimura[4], Tetsuro Matano[1,2] and Saori Matsuoka[2]

[1]Division for AIDS Vaccine Development, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, [2]AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, [3]Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, and [4]Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

ABSTRACT

Major histocompatibility complex class I (MHC-I)-restricted CD8$^+$ cytotoxic T lymphocyte (CTL) responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. In particular, Gag-specific CTL responses have been shown to exert strong suppressive pressure on HIV/SIV replication. Additionally, association of Vif-specific CTL frequencies with *in vitro* anti-SIV efficacy has been suggested recently. Host MHC-I genotypes could affect the immunodominance patterns of these potent CTL responses. Here, Gag- and Vif-specific CTL responses during primary SIVmac239 infection were examined in three groups of Burmese rhesus macaques, each group having a different MHC-I haplotype. The first group of four macaques, which possessed the MHC-I haplotype *90-010-Ie*, did not show Gag- or Vif-specific CTL responses. However, Nef-specific CTL responses were elicited, suggesting that primary SIV infection does not induce predominant CTL responses specific for Gag/Vif epitopes restricted by *90-010-Ie*-derived MHC-I molecules. In contrast, Gag- and Vif-specific CTL responses were induced in the second group of two *89-075-Iw*-positive animals and the third group of two *91-010-Is*-positive animals. Considering the potential of prophylactic vaccination to affect CTL immunodominance post-viral exposure, these groups of macaques would be useful for evaluation of vaccine antigen-specific CTL efficacy against SIV infection.

Key words cytotoxic T lymphocyte, human immunodeficiency virus, major histocompatibility complex, simian immunodeficiency virus.

Virus-specific CD8$^+$ CTL responses are crucial for the control of HIV and SIV replication (1–5). CTLs recognize specific epitopes which are presented on the target cell surface by binding to the MHC-I molecule. There have been many reports indicating association of MHC-I (HLA class I) genotypes with rapid or delayed AIDS progression in HIV-infected people (6–8). For instance, most of the HIV-infected individuals possessing *HLA-B*57* have a better prognosis and smaller viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in control

Correspondence
Saori Matsuoka, AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel: 81 3 5285 1111; Fax: 81 3 5285 1165; e-mail: s-matsu@nih.go.jp

Received 11 May 2011; revised 18 July 2011; accepted 21 August 2011.

List of Abbreviations: CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IFN-γ, gamma interferon; MHC-1, major histocompatibility complex class I; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

of this virus (9, 10). Indian rhesus macaques possessing the MHC-I allele Mamu-B*17 tend to show smaller viral loads after SIVmac239 challenge (11). These findings imply possible HIV control by induction of particular effective CTL responses.

The potential of Gag-specific CTL responses to contribute to viral control was suggested by a cohort study indicating association of HIV control with the breadth of Gag-specific CTL responses (12). This was supported by an *in vitro* study indicating the ability of Gag-specific CTLs to respond rapidly to SIV infection (13). We previously developed a prophylactic AIDS vaccine using a Sendai virus vector expressing SIVmac239 Gag (14) and showed that Gag-specific CTL responses were responsible for vaccine-based SIV containment in a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-120-Ia* (15, 16). Furthermore, our recent study analyzing the potential of $CD8^+$ cells to suppress SIV replication *in vitro* suggested association of *in vitro* anti-SIV efficacy with numbers of Vif-specific CTL frequencies (17). We also found weaker correlation between anti-SIV efficacy and numbers of Nef-specific CTL frequencies. These results imply the potency of Gag- and Vif-specific (and possibly Nef-specific) CTLs in suppressing HIV/SIV replication.

The immunodominance patterns of these potent CTL responses could be affected by host MHC-I genotypes (18, 19). Better understanding of these MHC-I-associated CTL immunodominance patterns during primary HIV/SIV infection would contribute to elucidation of the interaction between viral replication and host CTL responses. In the present study, we examined whether Gag- and Vif-specific CTL responses are efficiently induced during primary SIVmac239 infection in three groups of Burmese rhesus macaques possessing different MHC-I haplotypes. One group did not induce Gag- or Vif-specific CTL responses, whereas the other two groups elicited Gag- and Vif-specific CTL responses efficiently. These groups of macaques would be useful for analysis of the impact of Gag- and Vif-specific CTL responses on SIV replication *in vivo*.

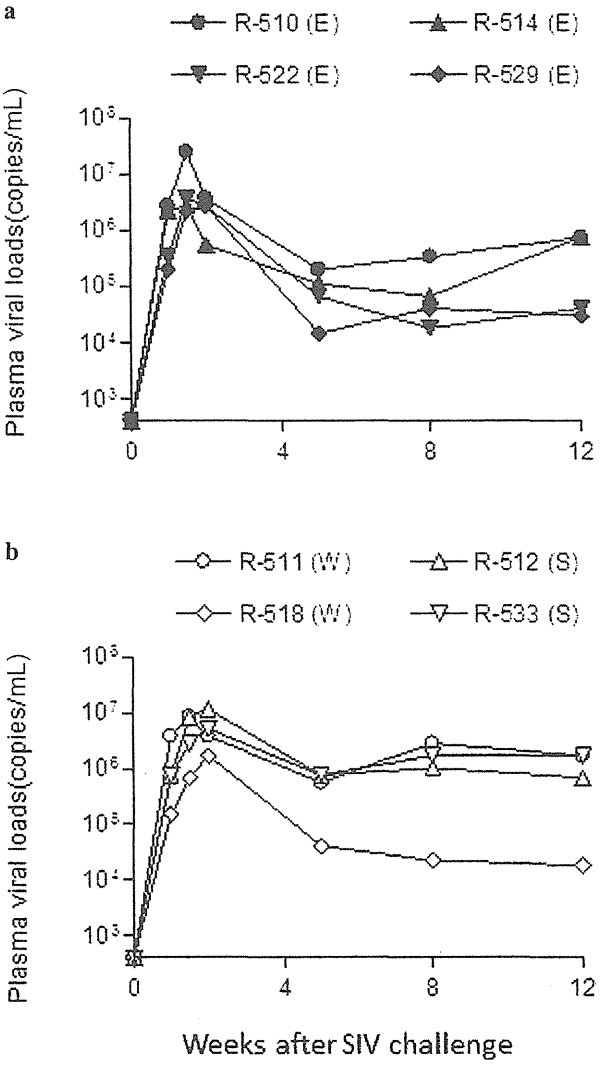

Fig. 1. Plasma viral loads after SIV challenge. (a) The first group of Burmese rhesus macaques, which possessed MHC-I haplotype *90-010-Ie* (R-510, R-514, R-522, and R-529) and (b) the second group, which possessed *89-075-Iw* (R-511 and R-518) and the third group, which possessed *91-010-Is* (R-512 and R-533) were challenged with SIVmac239. The viral loads (SIV gag RNA copies/mL) were determined as described previously (15).

MATERIALS AND METHODS

Animal experiments

Animal experiments using Burmese rhesus macaques (*Macaca mulatta*) possessing either the MHC-I haplotypes *90-010-Ie*, *89-075-Iw* or *91-010-Is* were performed in the Institute for Virus Research, Kyoto University, in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates. The MHC-I haplotypes of macaques were determined as described previously (20, 21). These animals were challenged intravenously with 1000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (22).

Analysis of virus-specific cytotoxic T lymphocyte responses

Virus-specific $CD8^+$ T-cell frequencies were measured by flow cytometric analysis of IFN-γ induction after specific stimulation as described previously (17). PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines pulsed with peptide pools using panels of overlapping peptides