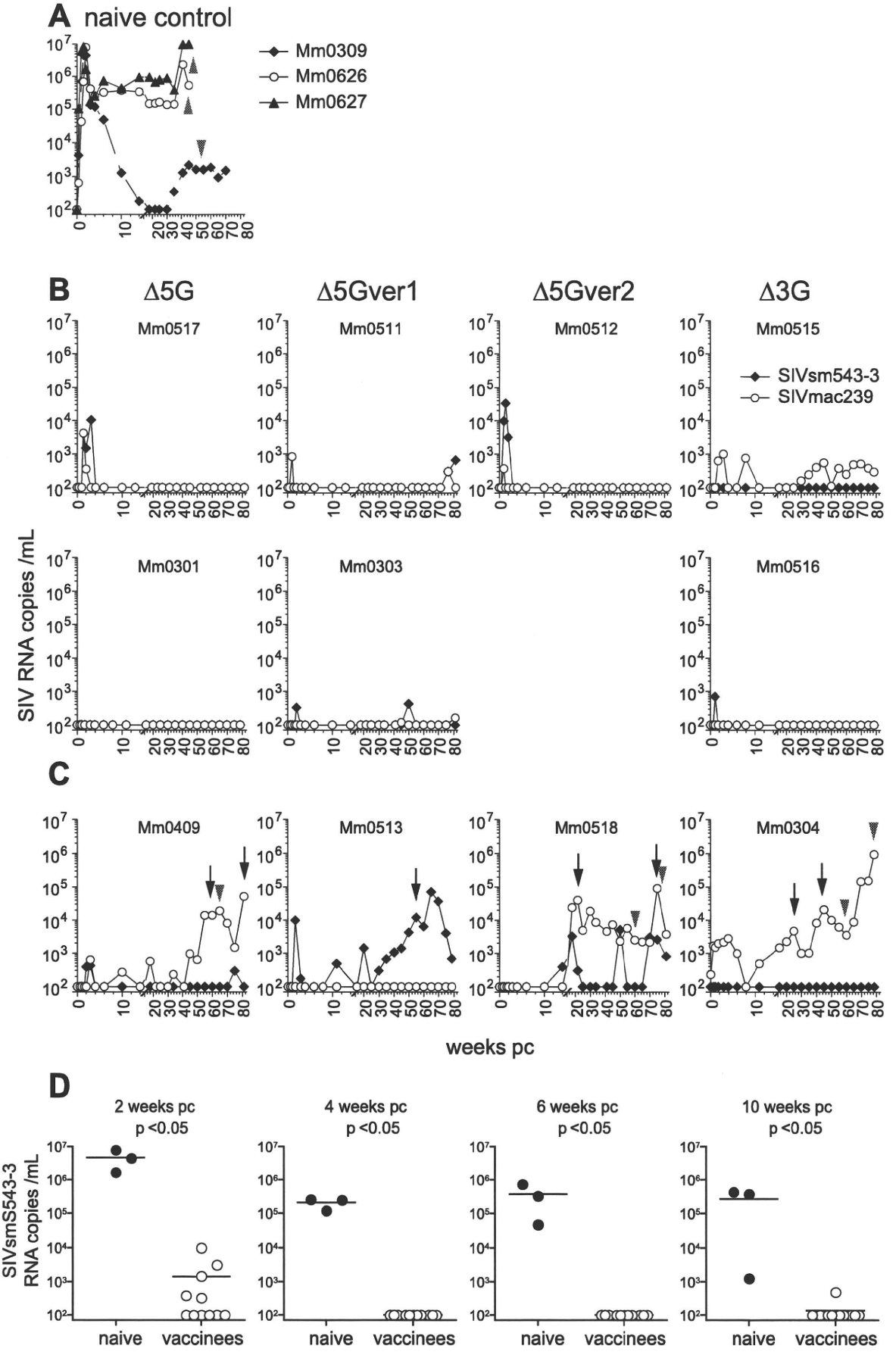
A naive control
Mm0309
Mm0626
Mm0627
B
Δ5G
Δ5Gver1
Δ5Gver2
Δ3G
Mm0517
Mm0511
Mm0512
Mm0515
SIVsm543-3
SIVmac239
Mm0301
Mm0303
Mm0516
SIV RNA copies /mL
C
Mm0409
Mm0513
Mm0518
Mm0304
weeks pc
D
2 weeks pc
p <0.05
4 weeks pc
p <0.05
6 weeks pc
p <0.05
10 weeks pc
p <0.05
SIVsmS543-3
RNA copies /mL
naive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

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

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

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

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

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

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

Adaptive immune responses in vaccinees

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

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

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

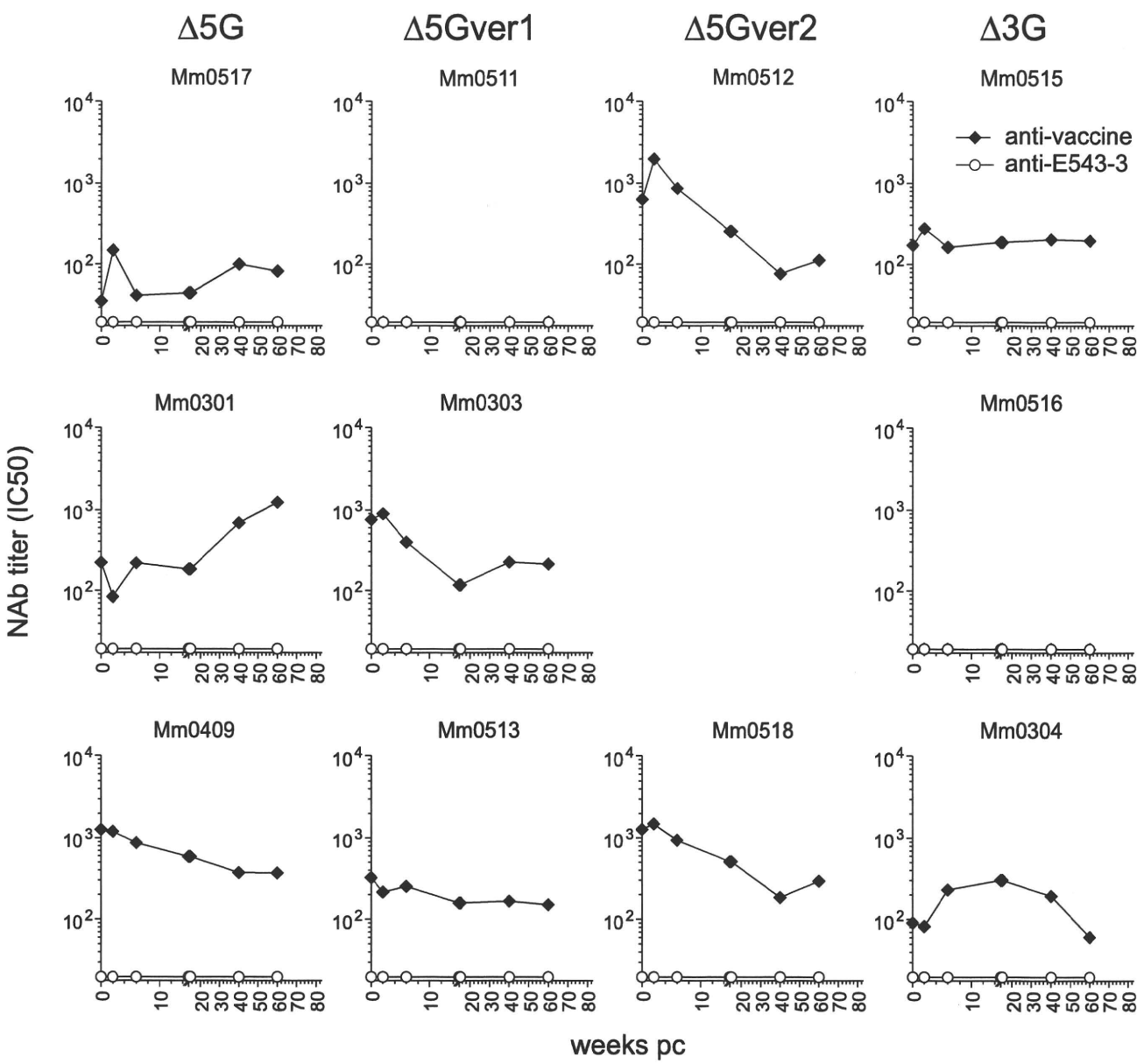

Figure 5. Neutralizing antibodies (NAb) response in the vaccine recipients. NAb responses against vaccine viruses (closed squares) and challenge virus (open circles) were measured in CEMx174/SIVLTR-SEAP system. NAb titers were indicated as the reciprocal of the dilutions of the plasma from the vaccinees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

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

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

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

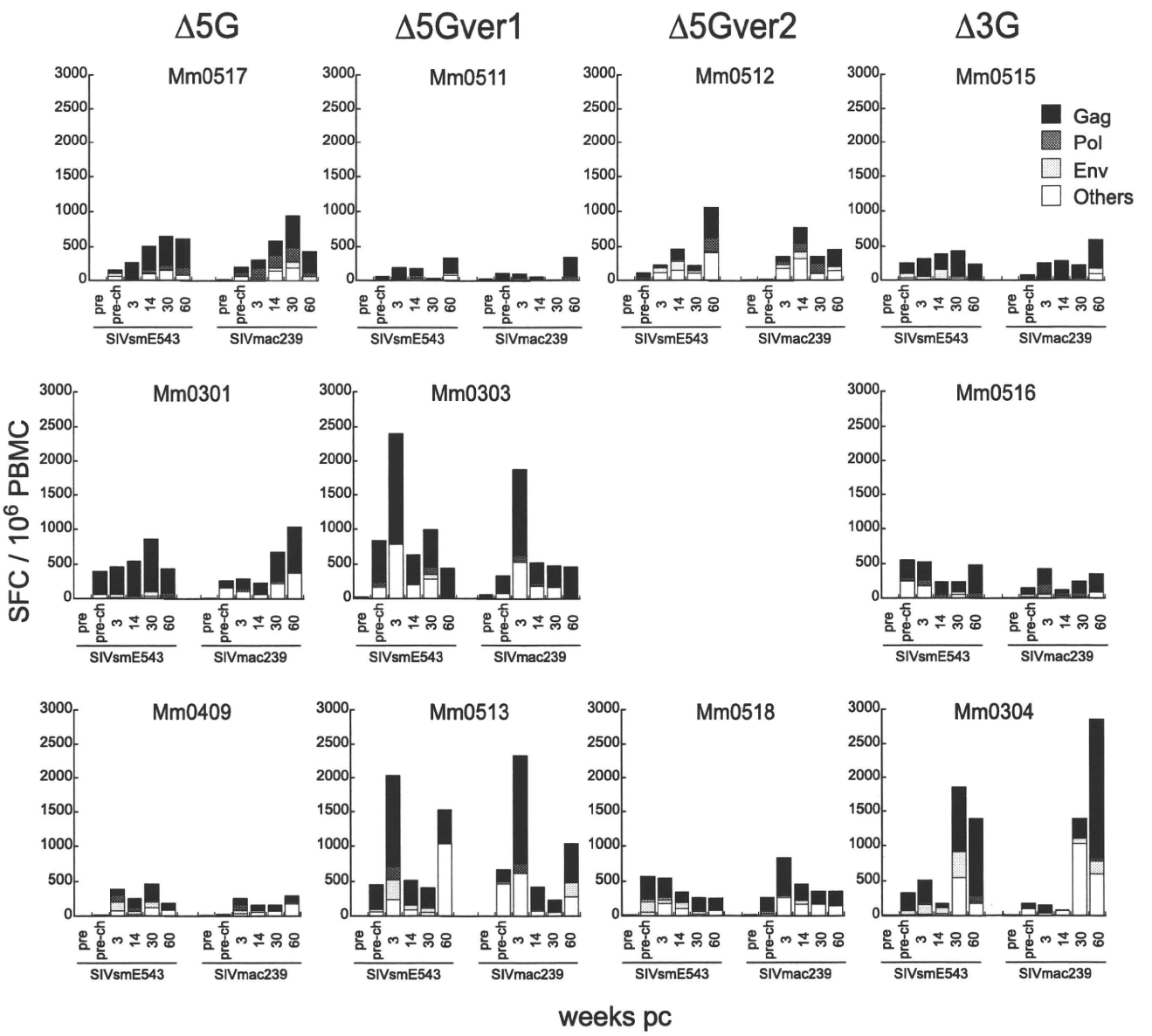

Figure 6. SIV specific cellular response in the vaccine recipients. SIV specific T cells were stimulated with overlapping peptides encompassing the viral proteins (Gag, Pol, Env, and Others: Vif, Vpr, Vpx, Tat, Rev, and Nef) of SIVmac239 and SIVsmE543-3 and the number of spot forming cells (SFC) per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

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

Discussion

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

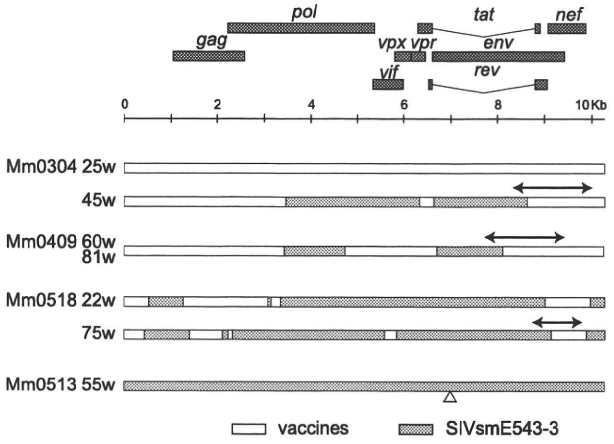

Figure 7. Recombination between vaccine and challenge virus. Nucleotide sequences of SIV fragments amplified by sets of nested PCR using primers based on SIVsmE543-3 or SIVmac239 to cover the entire SIV genome except the 5′ and 3′ terminal sequences (~100 bp). Representative sequences from integrated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

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

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

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

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

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

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

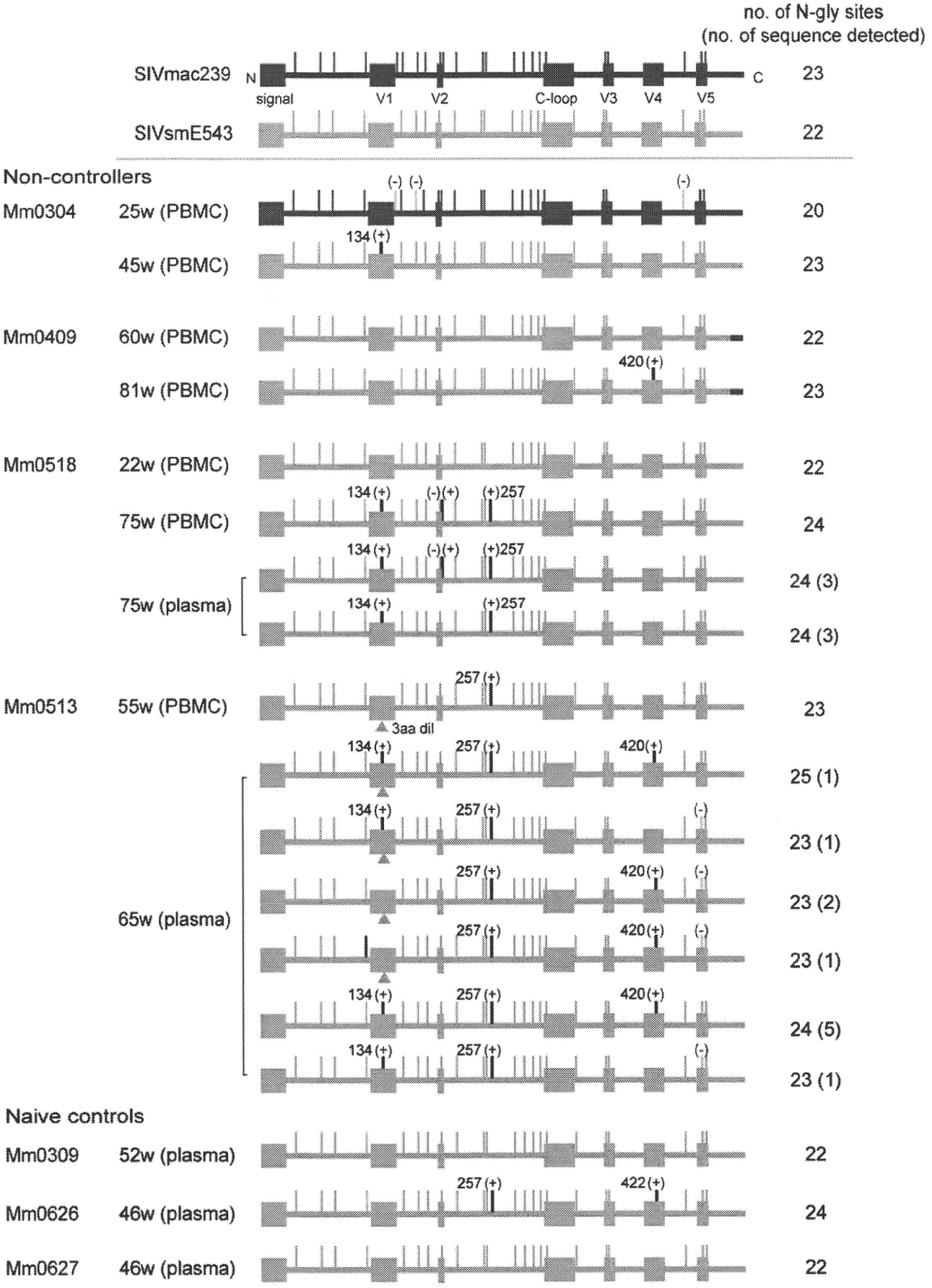
no. of N-gly sites
(no. of sequence detected)
SIVmac239
N
C
signal
V1
V2
C-loop
V3
V4
V5
23
SIVsmE543
22
Non-controllers
Mm0304
25w (PBMC)
(-) (-)
(-)
20
45w (PBMC)
134 (+)
23
Mm0409
60w (PBMC)
22
81w (PBMC)
420 (+)
23
Mm0518
22w (PBMC)
22
75w (PBMC)
134 (+)
(-)(+)
(+)257
24
75w (plasma)
24 (3)
24 (3)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

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

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

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

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

Materials and Methods

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

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

Attenuated vaccine viruses and challenge virus

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

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

Animals

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

Vaccination and challenge infection

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

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

Plasma viral load measurements

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

Sequencing of SIV RNA and proviral DNA

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

SIV specific T cell responses

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

Neutralization assay

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

Statistical analysis

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

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ORIGINAL PAPER

Diversity of MHC class I genes in Burmese-origin rhesus macaques

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Abstract Rhesus macaques (*Macaca mulatta*) are widely used in developing a strategy for vaccination against human immunodeficiency virus by using simian immunodeficiency virus infection as a model system. Because the genome diversity of major histocompatibility complex (MHC) is well known to control the immune responsiveness to foreign antigens, MHC loci in Indian- and Chinese-origin macaques used in the experiments have been characterized, and it was revealed that the diversity of MHC in macaques was larger than the human MHC. To further characterize the diversity of *Mamu-A* and *Mamu-B* loci, we investigated a total of 73 different sequences of *Mamu-A*, 83 sequences of *Mamu-B*, and 15 sequences of *Mamu-I* cDNAs isolated from Burmese-origin macaques. It was found that there were one to five expressing genes in each locus. Among the *Mamu-A*, *Mamu-B*, and *Mamu-I* sequences, 44 (60.2%), 45 (54.2%), and 8 (53.3%), respectively, were novel, and most of the other known alleles were identical to those reported from Chinese- or Indian-origin macaques, demonstrating a genetic mixture between the geographically distinct populations of present day China and India. In addition, it was found that a *Mamu* haplotype contained at least two highly transcribed *Mamu-A* genes, because multiple *Mamu-A1* cDNAs were obtained from one haplotype. These findings further revealed the diversity and complexity of MHC locus in the rhesus macaques.

Keywords Rhesus macaque · MHC · *Mamu-A* · *Mamu-B* · *Mamu-I* · Haplotype

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Introduction

The rhesus macaque (*Macaca mulatta*) is a member of the old world monkey. It is estimated that the ancestor of macaques was diverged from the human-chimpanzee ancestor approximately 25 million years ago (Stewart and Disotell 1998). The habitat of the rhesus macaque extends from Pakistan and India to the southern part of China

(Timmins et al. 2008), wider than that of the other nonhuman primates. It is known that the genome diversity in rhesus macaques is quite unique, because more than 60% of the rhesus macaque-specific expansions are found in the protein coding sequences (Gibbs et al. 2007). The increase in the gene copy number in the rhesus macaque, relative to that in humans, can also be observed in the major histocompatibility complex (MHC) locus (Gibbs et al. 2007).

The rhesus macaque is widely used as a nonhuman primate species model in biomedical researches for human diseases including acquired immunodeficiency syndrome (AIDS). Particularly, the development of vaccines against the human immunodeficiency virus (HIV) in part depends on the results of experiments using macaques, because the simian immunodeficiency virus (SIV) infection causes AIDS-like syndrome (Barouch et al. 2000; Schmitz et al. 1999; Yasutomi et al. 1993). Previous SIV challenge studies indicated association of MHC class I genotypes with rapid or delayed AIDS progression in rhesus macaques like HIV-1 infection in humans (Mothe et al. 2003; Yant et al. 2006; Loffredo et al. 2008; Reynolds et al. 2008).In addition, effective vaccination was associated with specific MHC class I alleles called as "elite controller" alleles, by which prevention of viral replication could be achieved in macaques challenged by SIVmac239 (Loffredo et al. 2007; Maness et al. 2008). In these experiments, macaques of Indian or Chinese origin have been widely used, and macaques from different regions such as Burma have also been used recently.

To evaluate the efficacy of SIV vaccination, it is necessary to characterize the MHC alleles because the presentation of antigenic peptides by MHC molecules to T cells, more specifically the binding of antigenic peptide to the MHC molecule, depends on the structure of the MHC allele. We have previously developed a reference strand conformation analysis-based typing system for *Mamu* class I genes and reported that the number of expressing genes varies among macaques of Burmese or Laotian origin; we could identify at least 16 different *Mamu* class I locus haplotypes that were composed of different numbers of *Mamu* class I genes (Tanaka-Takahashi et al. 2007). In addition, we reported that a haplotype of *Mamu* class I genes, *90-120-Ia*, exerted a protective vaccination against SIVmac239 challenge (Matano et al. 2004). Furthermore, it was revealed that one of highly expressed *Mamu-A* allele of the *90-120-Ia* haplotype, *Mamu-A1*065:01* (previously designated as *Mamu-A*90120-5*), encoded a Mamu-A molecule that could efficiently present a SIV-derived $Gag_{241-249}$ peptide to cytotoxic T cells from the vaccinated macaques (Tsukamoto et al. 2008).

The aim of present study was to define the allelic polymorphisms and haplotype diversity of the *Mamu* class I gene from Burmese-origin macaques.

Materials and methods

Animals

A total of 100 rhesus macaques from breeding colonies maintained in Japan were enrolled. Founders of colonies were captured in Myanmar or Laos, and the colonies were separately maintained. Macaque colonies were classified into seven groups based on their paternal lineages (90-120, 90-010, 90-030, 90-088, 89-002, 89-075, and 91-010F1) (Tanaka-Takahashi et al. 2007). The animal 91-010F1 was an offspring of 89-075.

Sequencing analysis of cDNAs from *Mamu* class I genes

Total cellular RNA was extracted from B lymphoblastoid cell lines established from the macaques by using RNAiso reagent (TaKaRa, Shiga, Japan). Oligo (dT)-primed cDNA was synthesized using Transcriptor reverse high fidelity transcriptase (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Full-length cDNAs for *Mamu* class I genes were amplified by polymerase chain reaction (PCR) using locus-specific primer pairs, as described previously (Tanaka-Takahashi et al. 2007), with a modification of primer pairs to those reported by Karl et al. (Karl et al. 2008): 5'MHC_UTR (5'-GGACTCAGAAT CTCCCCAGACGCCGAG) and 3'MHC_UTR_A (5'-CAG GAACAYAGACACATTCAGG) for *Mamu-A* locus and 5'MHC_UTR and 3'MHC_UTR_B (5'-GTCTCTCCAC CTCCTCAC) for *Mamu-B*, *-I* loci, using Phusion Flash DNA polymerase (Finzymes, Espoo, Finland). The PCR

Table 1 *Mamu* class I alleles found in Burmese-origin macaques

Loci	Number of analyzed macaques	Number of observed alleles	Novel alleles (number, %)		Known alleles (number, %)	
Mamu-A	100	73	44	60.2	29	39.8
Mamu-B	93	83	45	54.2	38	45.8
Mamu-I	93	15	8	53.3	7	46.7
Others (AG, F)	93	2	0	-	2	100
Total		173	97	56.1	76	43.9

Table 2 Alleles of *Mamu-A* locus identified in Burmese-origin macaques

Locus	Allele name	Novelty[a]	Accession Number[b]	Shared allele[c]	Number of animals	Identity to *Mafa* or *Mane* alleles[d]
A1	A1*003:01:03	Novel	AB496714		1	
A1	A1*003:08		AB444903	C	7	
A1	A1*003:10	Novel	AB444904		1	
A1	A1*004:01:02		AB444866	C	19	*Mafa-A1*004:02*
A1	A1*007:06:01	Novel	AB540211		2	
A1	A1*008:01:02	Novel	AB430443		11	
A1	A1*008:01:03	Novel	AB496711		1	
A1	A1*008:02	Novel	AB477383		2	
A1	A1*015:01		AB551785		2	
A1	A1*018:05		AB444927	I	1	
A1	A1*018:07	Novel	AB444928		11	
A1	A1*018:08	Novel	AB444926		6	
A1	A1*019:02		AB444900	C	2	
A1	A1*019:05		AB444901	C	1	
A1	A1*019:07	Novel	AB444899		2	
A1	A1*022:01		AB444895	C	1	
A1	A1*022:03	Novel	AB444894		7	
A1	A1*023:02	Novel	AB444874		4	
A1	A1*026:03		AB477385	C	1	
A1	A1*028:06	Novel	AB444924		1	
A1	A1*028:07:01	Novel	AB444923		3	
A1	A1*032:02	Novel	AB444933		13	
A1	A1*032:03	Novel	AB444934		4	
A1	A1*040:01		(AM295910)		1	
A1	A1*041:01		AB444931	C	1	
A1	A1*041:02		(EU429608)	C	1	
A1	A1*042:01	Novel	AB444868	C	2	
A1	A1*043:01		AB444869	C	7	
A1	A1*049:03		AB444880	C	2	
A1	A1*049:04	Novel	AB444881		2	
A1	A1*050:01		AB444889	C	7	
A1	A1*052:01		AB444890	C	3	*Mafa-A1*052:02*
A1	A1*056:02		AB477384	C	6	
A1	A1*056:02:02	Novel	AB444935		3	
A1	A1*065:01		AB444921	C	6	*Mafa-A1*065:04*
A1	A1*066:01	Novel	AB444888		14	
A1	A1*074:04:01	Novel	AB540213		1	
A1	A1*105:01	Novel	AB444898		1	
A1	A1*105:02	Novel	AB444896		11	
A1	A1*105:03	Novel	AB496716		2	
A1	A1*105:04	Novel	AB496709		1	
A1	A1*106:01	Novel	AB444875		1	
A1	A1*107:01	Novel	AB444887		9	*Mafa-A1*096:01*
A1	A1*108:01	Novel	AB444925		1	
A1	A1*109:01	Novel	AB444902		7	*Mafa-A1*097:01*
A1	A1*110:01	Novel	AB444884		4	
A1	A1*111:01	Novel	AB444886		1	
A1	A1*112:01	Novel	AB496717		1	
A1	A1*117:01:01	Novel	AB540212		2	

Table 2 (continued)

Locus	Allele name	Novelty[a]	Accession Number[b]	Shared allele[c]	Number of animals	Identity to *Mafa* or *Mane* alleles[d]
A1	A1*118:01:01	Novel	AB540214		1	
A2	A2*01:03	Novel	AB444917		15	
A2	A2*05:03:02		AB444910	C	2	
A2	A2*05:10		AB444907	I	2	
A2	A2*05:11		AB444909	I	7	
A2	A2*05:13		(AM295927)	C	1	
A2	A2*05:14		(AM295928)	C	1	
A2	A2*05:15:04	Novel	AB444914		3	
A2	A2*05:22		AB444911	C	1	*Mane-A2*05:18*
A2	A2*05:26		AB496715	C	2	
A2	A2*05:31	Novel	AB444908		2	
A2	A2*05:32:02	Novel	AB444920		2	
A2	A2*05:44	Novel	AB444912		1	
A2	A2*05:45	Novel	AB444915		2	
A2	A2*05:46	Novel	AB444913		4	*Mane-A2*05:03:01*
A3	A3*13:13	Novel	AB496712		4	
A4	A4*01:02:02	Novel	AB444879		3	
A4	A4*14:03		AB444876	C, I	15	
A4	A4*14:04		AB444878	C	1	
A5	A5*30:01:01		(AM295945)	C	1	
A5	A5*30:01:02		AB444882	C	1	
A5	A5*30:06	Novel	AB444883		2	
A6	A6*01:01		AB444938	C	1	
A6	A6*01:05	Novel	AB444937		4	

[a] New alleles are indicated as novel

[b] Nucleotide sequences were submitted to public database and can be obtained with the indicated accession number. The accession numbers in the parentheses indicated that the Mamu class I sequences were identical to those numbers which had been deposited previously by other investigators.

[c] Alleles found in Burmese-origin macaques were shared with macaques originated from the other region. *C* Chinese-origin macaques, *I* Indian-origin macaques

[d] Identical sequences found in *Mafa* or *Mane* alleles

program was composed of the following steps: denaturation at 98°C for 10 s; 25 cycles at 98°C for 1 s, 63°C for 5 s, 72°C for 20 s; and additional extension at 72°C for 1 min. The PCR products were cloned into pSTBlue-1 Perfectly Blunt vector (Novagen, WI, USA) according to the manufacturer's instructions. Both strands from 30 to 90 independent cDNA clones obtained from each macaque for each locus were sequenced by BigDye Terminator cycling system and analyzed in an ABI 3730 automated sequence analyzer (Applied Biosystems, CA, USA).

Data analyses and nomenclature for *Mamu* class I alleles

Nucleotide sequences of cDNAs were analyzed and aligned using Genetyx Ver. 8 software package (Genetyx Corp., Japan). When at least three clones from independent PCR or from different individuals showed identical sequences, we submitted the sequences to DNA Data Bank of Japan database and to the Immuno Polymorphism Database for nonhuman primate MHC (http://www.ebi.ac.uk/ipd/mhc/submit.html; Robinson et al. 2003) to obtain official nomenclature for novel alleles of *Mamu-A* and *-B* genes. Phylogenetic analysis of *Mamu-A* sequences corresponding to exon 2, 3 and a part of exon 4 obtained in this study was done by using Gentetyx Ver. 8 software package. *Mamu-A1* 001:01* was included in the analysis as a reference. Neighbor-joining trees were constructed with the Kimura 2 parameter method. Bootstrap values were based on 5,000 replications.

Results

Identification of *Mamu* class I alleles in Burmese-origin macaques

We analyzed cDNA clones obtained by RT-PCR for *Mamu-A* locus and *Mamu-B* locus (Table 1). When at least three

clones with identical sequences were obtained from two independent PCR for an individual or from at least two individuals, the nucleotide sequences were considered to be real and not artifacts. We identified 73 different *Mamu-A* sequences in 100 individuals. Among them, 44 (60.2%) were novel, whereas the other 29 (39.8%) were identical to those reported mainly from Chinese- or Indian-origin macaques (Table 2). In addition, 50 sequences were from *Mamu-A1*, while 14, 1, 3, 3, and 2 sequences were from *Mamu-A2*, *-A3*, *-A4*, *-A5*, and *-A6*, respectively (Table 2). A neighbor-joining analysis showed that the sequences from the same minor *Mamu-A* genes were clustered with each other (Fig. 1).

On the other hand, 83 *Mamu-B* alleles and 15 *Mamu-I* alleles were observed in 93 individuals. Among them, 45 (54.2%) and 8 (53.3%) were novel *Mamu-B* and *Mamu-I*

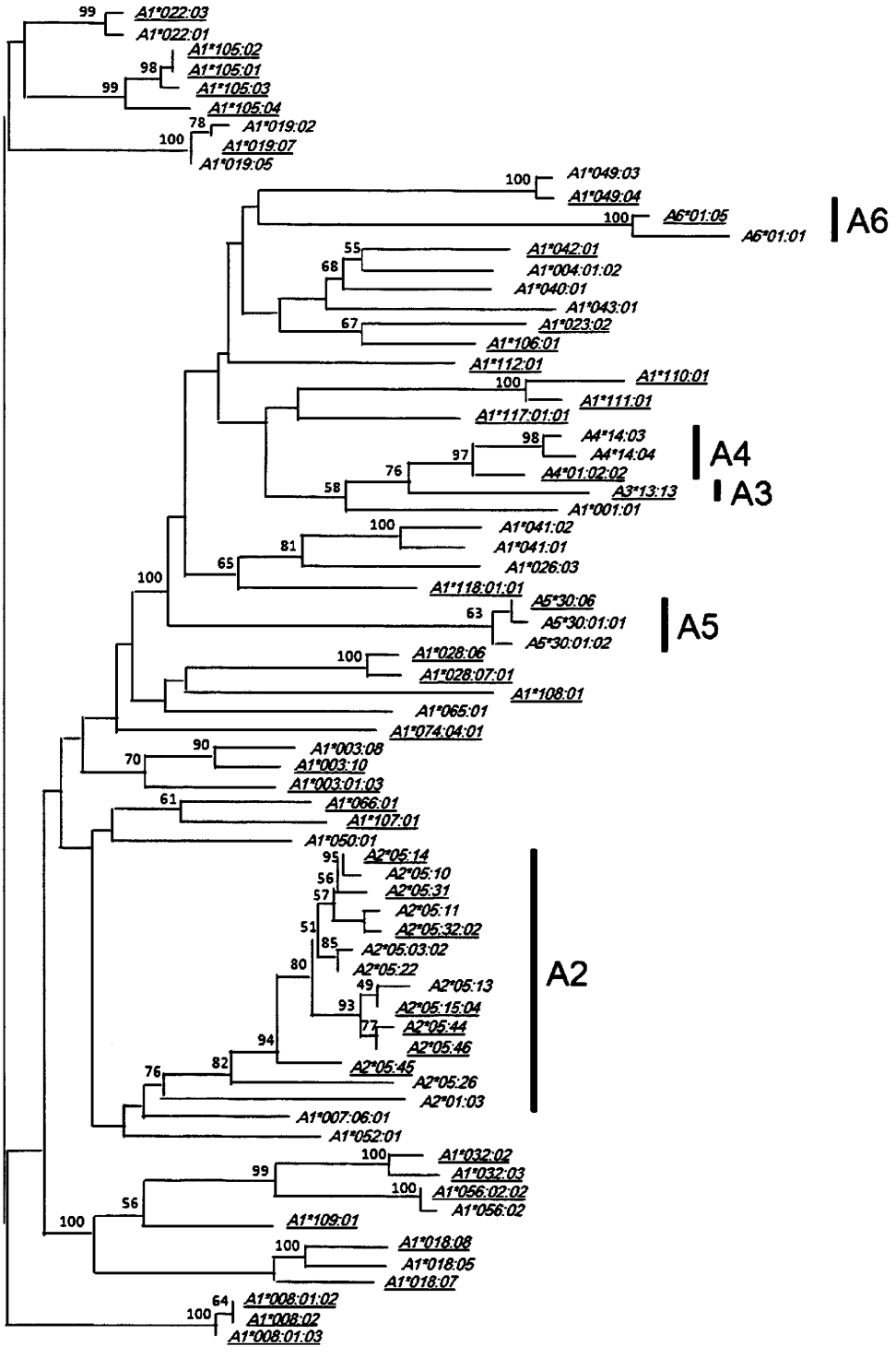

Fig. 1 Phylogenetic tree of *Mamu-A* alleles detected in this study. The tree was constructed using neighbor-joining method with bootstrap values of 5,000 replications. The values are indicated as percentages and those values less than 50% are not shown. *Mamu-A1* 001:01* was included in the analysis as a reference. The *Mamu-A* sequences with official nomenclature found in Burmese macaques are indicated, and novel alleles of Mamu-A genes are underlined. Clustering of alleles of minor *Mamu-A* genes, *Mamu-A2*, *-A3*, *-A4*, *-A5*, and *-A6* genes, are indicated by *vertical bars*

Table 3 Alleles of *Mamu-B* locus identified in Burmese-origin macaques

Locus	Allele name	Novelty[a]	Accession Number[b]	Shared allele[c]	Number of animals	Identity to *Mafa* or *Mane* alleles[d]
B	B*001:01:01		AB477408	I	12	
B	B*001:01:02		(AM902529)	C	6	
B	B*002:01		(U41833)	I	5	
B	B*003:01		(U41825)	C, I	2	
B	B*004:01		AB477405	I	11	
B	B*005:02		AB535753	I	14	
B	B*007:02		AB477409	C, I	33	
B	B*007:03		AB477412	C, I	1	
B	B*007:04:02	Novel	AB540183		2	
B	B*013:01		(AM902539)	C	1	
B	B*013:02:01	Novel	AB540185		1	
B	B*014:01		(AM902540)	C	1	*Mafa-B*105:01*
B	B*015:02		(AM902542)	C	1	
B	B*015:03:01	Novel	AB540186		2	
B	B*016:02:01	Novel	AB477395		9	
B	B*017:01		(AF199358)	I	2	
B	B*017:03		(AM902533)	C	8	
B	B*021:02		(AM902536)	C	1	
B	B*023:01		(AM902530)	C	2	
B	B*024:01		(AJ556881)	C, I	3	
B	B*026:02		AB477402	I	8	
B	B*028:02:01		(AM902532)	C	1	
B	B*029:03:01	Novel	AB540191		1	
B	B*036:03:01	Novel	AB477388		4	
B	B*037:01		AB477401	I	6	*Mafa-B*050:01*
B	B*038:01		(AJ556889)	I	1	
B	B*038:02:01	Novel	AB477391		3	
B	B*039:01		AB477411	C, I	12	
B	B*040:01:01	Novel	AB535751		8	
B	B*043:01		AB477403	C, I	14	
B	B*044:06:01	Novel	AB540205		1	
B	B*045:07:01	Novel	AB477389		5	*Mafa-B*012:01*
B	B*046:03:01	Novel	AB477397		2	
B	B*046:15		(EU915284)	I	1	
B	B*046:18:01	Novel	AB477398		2	
B	B*046:19:01	Novel	AB540193		1	
B	B*051:06:01	Novel	AB477387		2	
B	B*051:07:01	Novel	AB540206		1	
B	B*054:02:01	Novel	AB540194		5	
B	B*056:03:01	Novel	AB540195		2	
B	B*056:04:01	Novel	AB540207		2	
B	B*059:01		(AM902563)	C	1	
B	B*060:01		(EU669870)	I	1	
B	B*060:03		(EU934766)	I	1	
B	B*060:04:01	Novel	AB477394		4	
B	B*061:02		(AM902564)	C	3	
B	B*061:03	Novel	AB430442		7	
B	B*061:04:01	Novel	AB540196		10	*Mane-B*061:01*
B	B*063:02:01	Novel	AB540210		3	

Table 3 (continued)

Locus	Allele name	Novelty[a]	Accession Number[b]	Shared allele[c]	Number of animals	Identity to *Mafa* or *Mane* alleles[d]
B	B*063:02:02	Novel	AB540197		4	
B	B*063:04:01	Novel	AB477399		2	
B	B*063:05:01	Novel	AB540204		2	
B	B*066:01		AB477406	I	28	
B	B*066:02:01	Novel	AB540198		1	
B	B*068:04		(AM902571)	C	10	
B	B*069:01		(AF519898)	C, I	1	
B	B*069:06:01	Novel	AB540209		1	
B	B*069:07:01	Novel	AB540208		2	
B	B*070:02		(AM902575)	C	1	
B	B*071:01		(AJ489330)	I	2	
B	B*071:02:01	Novel	AB540199		1	
B	B*073:01		AB477404	C	4	
B	B*073:02:01	Novel	AB540200		1	
B	B*074:02		(AF219484)	C	1	
B	B*077:02		AB477410	C	1	*Mafa-B*110:01*
B	B*082:01		(EF580160)	C	1	
B	B*082:05:01	Novel	AB477396		5	
B	B*082:06:01	Novel	AB540201		2	
B	B*083:01		(EF580161)	C	2	
B	B*083:02:01	Novel	AB542052		1	
B	B*085:03:01	Novel	AB540202		5	
B	B*089:01		(EF580172)	C	11	
B	B*091:03	Novel	AB551786		2	
B	B*092:02:01	Novel	AB477386		7	
B	B*092:03:01	Novel	AB542053		1	
B	B*101:01:01	Novel	AB477400		3	
B	B*102:01:01	Novel	AB477392		10	
B	B*105:01:01	Novel	AB540184		1	*Mane-B*105:01*
B	B*124:01:01	Novel	AB540203		10	*Mane-B*124:01*
B	B*142:01:01	Novel	AB542050		1	*Mafa-B*023:02*
B	B*156:01:01	Novel	AB540192		1	
B	B*162:01:01	Novel	AB477390		3	
B	B*163:01:01	Novel	AB542051		2	
I	I*01:06:01		(EF580176)	C	2	
I	I*01:06:05		(EU934767)	I	4	
I	I*01:06:07		(FN396419)		1	*Mafa-I*01:11*
I	I*01:06:08	Novel	AB477416		12	
I	I*01:06:09	Novel	AB541976		3	*Mane-I*01:01:02*
I	I*01:06:10	Novel	AB541977		1	
I	I*01:07:01		AB477420	I	7	
I	I*01:08:01		(FJ009194)	I	13	
I	I*01:08:02		(GQ471888)	I	4	
I	I*01:09:01	Novel	AB477415		1	
I	I*01:18		(EF580175)	C	1	
I	I*01:20:02	Novel	AB477414		2	
I	I*01:22:01	Novel	AB477417		7	
I	I*01:23:01	Novel	AB477418		8	
I	I*01:24:01	Novel	AB477413		2	

Table 3 (continued)

Locus	Allele name	Novelty[a]	Accession Number[b]	Shared allele[c]	Number of animals	Identity to *Mafa* or *Mane* alleles[d]
F	F*01:03			I	3	
AG	AG*03:01:01			I	1	

[a] New alleles are indicated as novel

[b] Nucleotide sequences were submitted to public database and can be obtained with the indicated accession number. The accession numbers in the parentheses indicated that the Mamu class I sequences were identical to those numbers which had been deposited previously by other investigators.

[c] Alleles found in Burmese-origin macaques were shared with macaques originated from the other region. *C* Chinese-origin macaques, *I* Indian-origin macaques

[d] Identical sequences found in *Mafa* or *Mane* alleles

alleles, respectively. The other *Mamu-B* and *Mamu-I* sequences were identical to those reported from Chinese- and/or Indian-origin macaques (Table 3).

Mamu class I haplotypes observed in Burmese-origin macaques

From the cDNA analyses of genetically related macaques, we could identify the *Mamu-A* and *Mamu-B* sequences comprising 13 different haplotypes from seven paternal lineages (haplotype 'w' was shared by 89-075 and its offspring 91-010F1) and eight other haplotypes in the colonies; the *Mamu* class I haplotype consisted of one to three expressing *Mamu-A* genes and one to five expressing *Mamu-B* (including Mamu-I) genes, confirming that the number of expressed *Mamu* class I genes varied with the haplotype (Table 4). Examples of family pedigrees are shown in Fig. 2. Although usually only one *Mamu-A1* allele could be identified in the haplotypes, the *90-120-a* haplotype carried two different *Mamu-A1* alleles, which was confirmed by the analysis of family pedigree (Fig. 2a). In addition, *Mamu-B*001* alleles were tightly linked to a *Mamu-B*007* allele (Table 4).

Table 4 *Mamu* class I haplotypes identified in Burmese-origin macaques

Founder Lineage[a]	Haplotype	Major Mamu-A (A1)	Minor Mamu-A	Mamu-B
90-120	a	A1*043:01, A1*065:01		B*061:03, B*068:04, B*089:01
	b	A1*018:08	A2*05:31	B*036:03:01, B*037:01, B*043:01, B*162:01:01,
90-010	d	A1*032:02		B*004:01, B*102:01:01
	e	A1*066:01		B*005:02, B*040:01:01
90-030	g	A1*105:02	A2*05:11	B*066:01
	h	A1*004:01:02	A4*14:03	B*043:01, B*092:02:01
90-088	j	A1*008:01:02		B*007:02, B*039:01
	k	A1*018:08	A2*05:45	B*001:01:01, B*007:02
89-002	p	A1*018:07	A2*01:03, A4*14:03	B*001:01:01, B*007:02
	q	A1*107:01		B*016:02:01
91-010F1	s	A1*003:08		B*023:01, I*01:08:01
	w	A1*022:03	A4*01:02:02	B*001:01:02, B*007:02, B*017:03
89-075	w	A1*022:03	A4*01:02:02	B*001:01:02, B*007:02, B*017:03
	v	A1 *109:01	A3*13:13	B*054:02:01, B*061:04:01, B*063:02:02, B*068:04, B*124:01:01
R428	i	A1*050:01	A2*05:11	B*066:01
R360	o	A1*028:07:01		B*056:04:01, B*066:01
R236	r	A1*049:03	A2*05:22	B*001:01:02, B*007:02, B*017:03
95-014	f	A1*066:01	A2*05:14, A5*30:01:01	B*005:02
R487	m	A1*018:08	A2*05:31	B*026:02, B*045:07:01, B*051:06:01
R252	t	A1*032:03	A2*05:14, A5*30:01:01	B*005:02
R446	u	A1*004:01:02		B*026:02, B*043:01, B*073:01
R220	c	A1*050:01		B*063:02:01, B*066:01

[a] ID of founder in which each Mamu class I haplotype was found

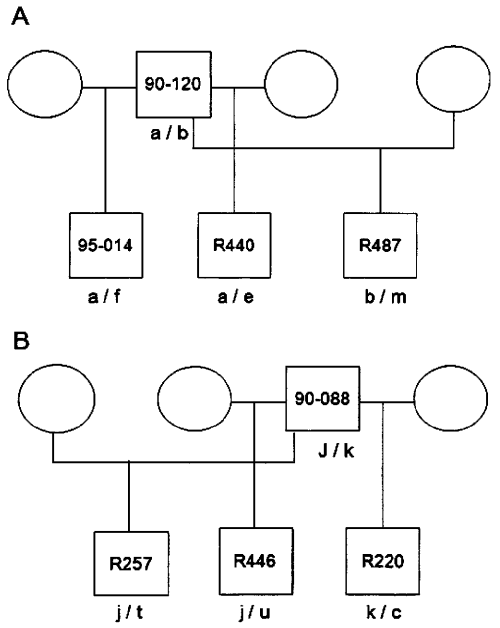

Fig. 2 Segregation of *Mamu* class I haplotypes in the pedigrees of macaques. Pedigree information and haplotype information are indicated along with ID of macaques. A. *Mamu* class I haplotypes of *a* and *b* in the parent (90-120) were segregated to its offspring 95-014, R440, and R487. B. *Mamu* class I haplotypes of *j* and *k* in the parent (90-088) were segregated to R257, R446, and R220. The *Mamu* class I alleles composing the indicated haplotypes are listed in Table 4

Discussion

The rhesus macaque is widely used in the experimental design for developing a vaccine against HIV. Indian-origin macaques are well characterized as a model system and it has been reported that there are several "elite controller" alleles such as *Mamu-A*001* and *Mamu-B*017*, with which most macaques showed lower viral loads after SIVmac239 challenge (Friedrich et al. 2004). In this study, we did not observe *Mamu-A1*001* in Burmese-origin macaques, while we previously reported that a group of animals carrying the MHC class I haplotype 90120a ('a' haplotype designated in this study, Table 4) showed vaccine-based control of SIVmac239 replication (Matano et al. 2004). This haplotype contains *Mamu-A*065:01* (previously noted as *Mamu-A*90120-5*) allele, and cytotoxic T lymphocyte (CTL) responses specific for an SIVmac239 $Gag_{241\text{-}249}$ (SSVDEQIQW) epitope restricted by this *Mamu-A1* allele are responsible for the SIV control in the vaccinated macaques carrying the 90120a haplotype (Kawada et al. 2008). Interestingly, the SIV $Gag_{241\text{-}249}$ epitope is overlapped with a HLA-B*5701-restricted HIV-1 $Gag_{240\text{-}249}$ epitope, TW10 (TSTLQEQIAW), and TW10-specific CTL responses have also been indicated to exert strong suppression on HIV-1 replication resulting in lower viral loads (Tsukamoto et al. 2008; Goulder and Watkins 2008).

Among 73 *Mamu-A* sequences detected in this study, only four sequences were reported to be found in the Indian-origin macaques. In clear contrast, 25 *Mamu-A* sequences were also found in the Chinese-origin macaques, implying that the genetic background of Burmese-origin macaques might be closer to Chinese-origin macaques than to Indian-origin macaques. However, 27 and 25 *Mamu-B* sequences were identical to those reported in Chinese- and Indian-origin macaques, respectively, demonstrating that Burmese-origin macaques represent a mixture of geographically distinct Chinese- and Indian-origin macaque populations. In addition, more than half of *Mamu* class I alleles found in this study were novel, indicating that the regional difference in MHC allelic distribution exists similar to that in human HLA. Because the habitat of Burmese-origin rhesus macaques is overlapped in part with the habitat of crab-eating macaques (cynomolgus rhesus, *Macaca fascicularis*) and Southern pig-tailed macaques (*Macaca nemestrina*), it is interesting to investigate whether the identical sequences to *Mamu* class I alleles would be frequently found in *Mafa* or *Mane* class I alleles. As shown in Tables 2 and 3, about 10% of *Mamu* class I alleles had identical sequences to equivalent *Mafa* or *Mane* class I alleles, as has been observed in the other macaque populations (Campbell et al. 2009; Otting et al. 2009), demonstrating that the frequency of shared MHC class I alleles was relatively constant in different populations of macaques.

The *Mamu* locus is known to be composed of multiple copies of polymorphic DNA sequences (Daza-Vamenta et al. 2004; Kulski et al. 2004); for example, *Mamu-A* locus encodes for a major and highly transcribed *Mamu-A1* and other minor *Mamu-A2*, *-A3*, *-A4*, *-A5*, *A6*, and *-A7* with relatively low transcription (Otting et al. 2004, 2007). In this study, we identified two different *Mamu-A1* alleles on one haplotype, *Mamu-A1*043:01* and *Mamu-A1*065:01* on the haplotype *90120-a*, which was confirmed by the segregation study of 90-120 family (Fig. 2a). In the phylogenetic tree of *Mamu-A* sequences, *Mamu-A1*043:01* and *Mamu-A1*065:01* alleles were classified into the *Mamu-A1* allele group (Fig. 1). These data showed the presence of *Mamu-A* haplotype carrying multiple major *Mamu-A1*, albeit that it might be a rare exception.

On the other hand, we deduced that some *Mamu-A1* alleles could not be well amplified by the PCR primer pair used in this study. For instance, *Mamu-A1*065:01* in the "a" haplotype (90-120 lineage, Table 4) and *Mamu-A1*003:08* in the "s" haplotype (91-010F1 lineage, Table 4) could not be well amplified with the primer-set of 5'MHC_UTR and 3'MHC_UTR_A. On the contrary, *Mamu-A1*004:01:02* in the "h" haplotype (90-030 lineage, Table 4) and *Mamu-A1*10:701* in the "q" haplotype (89-002 lineage, Table 4) were amplified more efficiently with this primer pair than the other primer pair reported previously (Tanaka-Takahashi et al. 2007). These observations raised a possibility that there might be further copy

number variations in the *Mamu* class I loci. It appears that a higher number of highly transcribed and expressed MHC alleles on a haplotype would be desirable, when the immunological role in antigen presentation after viral infection is considered, because the multiple MHC alleles will enable one to present more number of antigenic peptides. However, the presence of highly transcribed and expressed multiple MHC alleles could lead to multiple holes in the antigen recognition through elimination of T cells recognizing self-antigenic peptides or foreign antigenic peptides mimicking self-antigens. In this regard, it should be noted that the transcription levels of *Mamu-B* alleles, as estimated by the number of clones isolated from each macaque, were not so different from one another. We found that several *Mamu-B* alleles on the specific haplotypes, such as "b" haplotype (90-120 lineage) and "v" haplotype (89-075 lineage), showed similar transcription levels, although their expression levels might be moderate. However, because Rosner et al. reported that cell surface expression of Mamu molecules encoded by several *Mamu-B* alleles was weak at the similar expression level to that of *Mamu-A4* (Ronser et al. 2010), there might be a group of minor *Mamu-B*, indicating that further analyses will be required to decipher the complexity of *Mamu-B* locus.

It is worth noting that we observed a link between *B*001:01* and *B*007:02* in four different haplotypes (Table 4). It was reported that *B*001:01* and *B*007* were common in Indian- and Chinese-origin macaques and that a haplotype including these alleles, *Mamu-B*001, B*07*, and *B*030:02*, was frequently found in both populations (Otting et al. 2008). However, that *Mamu-B*030:02* or related allele was not found in Burmese-origin macaques suggested that the distance between *Mamu-B*001* and *B*07* was closer than that to *Mamu-B*030:02*.

In this study, we sequenced 30-90 clones for each locus obtained from each macaque. As has been described (Karl et al. 2008; Otting et al. 2007, 2004), picking up from 16 to 88 clones was enough to detect major *Mamu* class I alleles, for example, *Mamu-A1* alleles. Therefore, we hoped to isolate the major *Mamu-A1* alleles from all individuals in this study. On the other hand, there were only nine out of 21 haplotypes carrying a *Mamu-A2* allele in this study, although Bassinger et al. (2008) reported that 75% of Chinese-origin macaques carried at least one *Mamu-A2* allele. We could not exclude a possibility that our cDNA cloning strategy might be insufficient to detect *Mamu-A* genes with low expression, such as *Mamu-A2*. Alternatively, *Mamu-A* haplotypes not carrying *Mamu-A2* might be prevalent in Burmese-origin macaques. In addition, the number of *Mamu-I* alleles detected in this study was much less than that of *Mamu-B* alleles, which is consistent with the results in a previous report (Urvater et al. 2000).

In conclusion, we characterized the diversity of *Mamu* class I genes in the Burmese macaques, which showed, only in part, a similarity to Chinese- and Indian-origin macaques. Because the *Mamu-A1* gene is responsible for exerting the classical antigen presentation function (Chu et al. 2007; Sidney et al. 2000), characterization of the *Mamu-A* and *Mamu-B* alleles in Burmese-origin macaques will provide us with essential information in designing the vaccination experiments against SIV.

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Paradigm change in immune correlation: cellular or humoral?

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"Although the importance of the induction of broadly neutralizing antibodies by vaccines cannot be denied, cellular immunity-targeted candidate vaccines ... should also be clinically tested."

Current status of HIV/AIDS vaccine development

Recently, considerable progress has been achieved in the field of HIV vaccine development [1,2], and it has been suggested that the immune correlates of protection against viral infection comprise a complicated combination of viral and host immunological and genetic factors [3]. It is well known that some HIV-1-infected individuals do not exhibit disease progression as long as HIV-1 replication is well controlled by host immunity [4]. Despite great efforts to understand the immune status of such long-term nonprogressors and macaques immunized with live-attenuated simian immunodeficiency virus (SIV) vaccine, we still suffer from lack of sufficient knowledge on potential immune correlates of protection. In addition, the structural features and variability of the envelope (Env) glycoprotein (gp) of HIV-1 are responsible for our inability to elicit potent broadly neutralizing antibodies against HIV-1 by active immunization.

History of HIV vaccine efficacy trials

The history of efficacy trials for preventive HIV vaccines is marked by a series of paradigm shifts in immune correlation. First, Vaxgen Co. (CA, USA) tested a genetically engineered surface Env gp120 vaccine in humans. Despite induction of effective virus-neutralizing antibodies during the initial phases of the trial, a large-scale Phase III trial revealed the ineffectiveness of the vaccine [5]. This failure changed the strategy of HIV vaccine research from antibodytargeted to cell-mediated immunity-targeted. Considering the cell-associated features of HIV-1, cell-mediated immunity, especially that conferred by virus-specific cytotoxic T lymphocytes (CTLs), should be an important arm of the host immune system in regards to HIV infections. Indeed, it has been suggested that immunodeficiency virus-specific cellular immunity effectively controls viral replication during the natural course of infection [6,7]. Based on these considerations, various vaccine modalities, including live viral vectors and DNA, have been tested to elicit strong CTL and type 1 T-helper cell responses in nonhuman primate models. Although the DNA vaccine was not sufficiently immunogenic in macaques, boosting in DNA-primed individuals with viral vector vaccines, such as vaccinia virus [7], Sendaivirus [8] and adenovirus [9], amplified CTL responses and resulted in effective control of immunodeficiency virus replication. Among such viral vectors, vaccination with adenovirus type 5 (Ad5) elicited the strongest CTL induction. However, in 2007, the Merck (NJ, USA) STEP trial testing the efficacy of a recombinant (r) Ad5 vaccine was discontinued at Phase IIb because the vaccine failed to provide protective immunity [10]. Instead, the vaccinated group showed a significantly higher HIV-1 infection rate than the placebo group, which indicated that rAd5 immunization

EXPERT REVIEWS

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