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Mucosal prior to systemic application of recombinant adenovirus boosting is more immunogenic than systemic application twice but confers similar protection against SIV-challenge in DNA vaccine-primed macaques

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ABSTRACT

We investigated the immunogenicity and efficacy of a bimodal prime/boost vaccine regimen given by various routes in the *Simian immunodeficiency virus* (SIV) rhesus monkey model for AIDS. Twelve animals were immunized with SIV DNA-vectors followed by the application of a recombinant adenovirus (rAd5) expressing the same genes either intramuscularly (i.m.) or by oropharyngeal spray. The second rAd5-application was given i.m. All vaccinees plus six controls were challenged orally with SIVmac239 12 weeks post-final immunization.

Both immunization strategies induced strong SIV Gag-specific IFN-γ and T-cell proliferation responses and mediated a conservation of $CD4^+$ memory T-cells and a reduction of viral load during peak viremia following infection. Interestingly, the mucosal group was superior to the systemic group regarding breadth and strength of SIV-specific T-cell responses and exhibited lower vector specific immune responses. Therefore, our data warrant the inclusion of mucosal vector application in a vaccination regimen which makes it less invasive and easier to apply.

Introduction

Inducing a broad, durable and protective immunity is the primary aim of any AIDS vaccine. A wide range of vaccine vectors and delivery strategies have been tested in pre-clinical studies using simian immunodeficiency virus (SIV)-infected monkeys (Casimiro et al., 2004; Doria-Rose et al., 2003) or mice (McGettigan et al., 2006, 2001). The monkey model is the most relevant for such studies because it provides an opportunity to test both protective vaccine candidates (reviewed in McMichael, 2006) and chemo-prophylactic regimens (Van Rompay et al., 2006). Despite suggested caution particularly in the light of the failed human STEP HIV-1 Merck vaccine trial (Sekaly, 2008), adenoviral vectors generated highly potent and broad immune responses (Malkevitch et al., 2006, 2003). Of these, replication defective human adenovirus serotype 5 (rAd5) vectors expressing SIV proteins are frequently employed since they elicit a strong immune response against the product of the gene incorporated (Shiver et al., 2002). Pre-existing immunity to adenoviral vectors may negate its overall efficacy (Kostense et al., 2004; Sumida et al., 2005; Thorner et al., 2006), and is to blame in part for the failure of the clinical vaccine trial involving rAd5-vectors (Sekaly, 2008). However, these deficiencies can be overcome, including heterologous prime-boost regimes (Casimiro et al., 2003, 2004, 2005; Thorner et al., 2006). Using a similar vector design, we previously demonstrated strong anamnestic SIV-specific interferon (IFN)-γ responses capable to significantly limit viremia in vaccinated macaques after challenge (Suh et al., 2006). One critical aspect in the design of an effective vaccine is the development of a memory T-cell response. Recent studies in the SIV monkey model have shown that T-cell memory consists of distinct populations of central and effector memory cells (Pitcher et al., 2002). Here we defined the central memory T-cells as $CD4^+95^+29^+28^+$, thereby combining two previous definitions of memory T-cell markers (Picker et al., 2006; Sopper et al., 1997).

Interleukin-15 (IL-15) is a pleiotropic cytokine which has been used in vaccination and therapeutic studies with ambivalent results (Boyer et al., 2007; Demberg et al., 2008; Halwani et al., 2008; Hryniewicz et al., 2007; Mueller et al., 2008). Given after SIV-infection IL-15 expands both the $CD4^+$ and $CD8^+$ effector memory T-cell compartments (Picker et al., 2006) but it is also known to be a potent regulator of B- and

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natural killer cell proliferation (Waldmann, 2006). To enhance the cellular immune response IL-15 was included in our DNA prime rAd5 booster regimen. In addition, the DNA prime was supplemented by a synthetic immunostimulatory peptide which attracts human dendritic cells (Yang et al., 2002) and selectively enhances vaccine induced $CD8^+$ T-cell responses in mice (Lee et al., 2005).

Compared to the human STEP study regimen (Sekaly, 2008), our vaccine approach using the macaque model for AIDS, was completely different. First, we utilized E1 and E3 deleted rAd5, while only E1-deleted rAd5 was used in the STEP study. Second, we applied a heterologous prime-boost regimen comprising multigenic DNA-prime and recombinant rAd5 boost. We modified our previous regimen (Stahl-Hennig et al., 2007b) by using codon-optimized SIV DNA plus human IL-15 DNA followed by boosting with recombinant rAd5 expressing the same SIV genes as well as IL-15. Third, in contrast to the STEP study, we focused on the impact of mucosal immunization on vaccine efficacy. One study arm combined both mucosal and systemic administration of the rAd5 vaccine and was compared to a group receiving the vaccines systemically only. Following an oral challenge with SIVmac239 both vaccine groups revealed substantial protection as demonstrated by lower levels of peak viremia and better conservation of the central memory T-cell population compared to control animals. Furthermore, a combination of mucosal and systemic rAd5 immunization was more immunogenic than rAd5 given systemically alone in DNA vaccine-primed macaques.

Results

Strongest SIV-specific T-cell proliferation and Gag-specific IFN-γ ELISpot responses in the mucosal vaccine group

T-cell proliferation responses in blood were determined in all animals on the day of immunization and every 2 or 4 weeks, starting at 8 weeks after the first immunization. Proliferative activity was weak or undetectable either after DNA priming alone (data not shown) or after the first booster immunization with rAd5. Only after the second booster immunization we were able to demonstrate rising proliferative responses in the mucosal group by week 40 (Fig. 1A). On the day of challenge at week 44, these responses developed strongly in the mucosal group (mean SI = 11.5) compared to the systemic group (mean SI = 4.5; $p = 0.041$). Two weeks after challenge, T-cell proliferation responses dropped in all vaccinees, reappearing 2 weeks later although these could not be sustained thereafter.

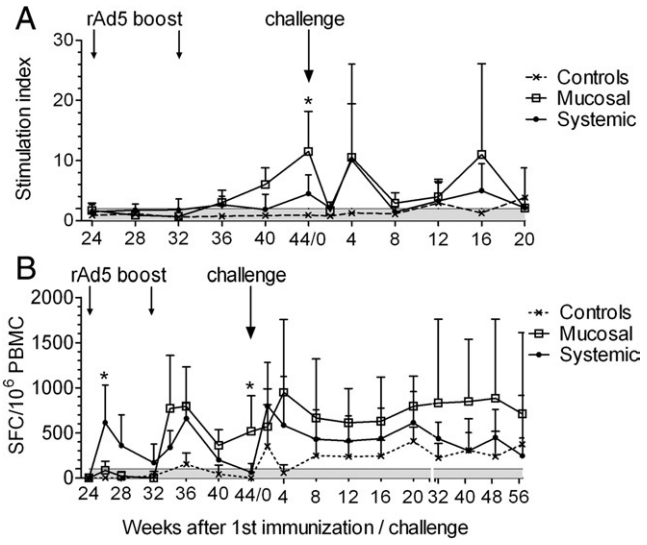

Fig. 1. T-cell proliferation responses against whole inactivated SIV (A) and SIVgag-specific IFN-γ ELISpot response profiles (B) during immunization and after challenge. The arrows indicate the rAd5 immunizations and day of challenge. The asterisk depicts a statistical difference ($p < 0.05$) between the vaccine groups.

Strongest SIV Gag-specific IFN-γ ELISpot responses in the mucosal group on the day of challenge

SIV Gag IFN-γ ELISpot responses peaked in the systemic group 2 weeks after first rAd5-boost (Fig. 1B, week 26) and were significantly higher than those in the mucosal group ($p = 0.009$). For the mucosal group these responses only peaked after the second rAd5-administration given i.m. while no significant responses were detected after the first boosting given mucosally in this group. Cumulative ELISpot responses against all peptides analyzed were relatively uniform for animals within the systemic and mucosal groups after the second boost (Fig. 2A) and remained so even for the relatively strong Gag-responses. Comparing means of all and Gag alone ELISPOT responses for each group revealed no significant difference between the two vaccine groups 2 weeks after the second boost (Fig. 2C). On the day of challenge these IFN-γ responses had declined in both groups but were significantly higher in the mucosal than in the systemic group (Fig. 2D). Our results also revealed that the mucosal group reacted against a wider spectrum of different SIV-peptide pools at challenge compared to the systemic group (Fig. 2B). After challenge, SIV Gag INF-γ ELISpot levels were significantly higher for vaccinees than control animals at 4 wpc but were not significantly different between the two vaccine groups. Moreover, these ELISPOT values remained higher in the mucosal than in the systemic group during the whole post-infection phase (Fig. 1B).

Collectively, these observations suggest a higher and more diverse immune response in the mucosal group.

Higher SIV-specific IgG binding and neutralizing responses in the mucosal group contrasting limited serum IgA in both groups

Animals of the mucosal group receiving the rAd5-boost first orally and then systemically developed higher SIV Gag p27 and envelope gp130 binding-antibody responses on the day of challenge when compared to the group boosted twice systemically. However, these differences were not significant between the vaccine groups (data not shown).

SIVmac251 neutralizing antibody (nAb) titers appeared in the systemic group 4 weeks after the first rAd5 vaccination (Fig. 3A) whereas in the mucosal group nAb titers were not present after oral immunization, but observed only after systemic boosting. Interestingly, on the day of challenge the mean nAb titer was significantly higher in the mucosal than in the systemic group. In contrast, neutralization antibodies against SIVmac239, which is more resistant to neutralization than SIVmac251, were undetectable during the immunization period. As a measure of mucosal immune responses, we examined the presence of SIV Env- and Gag-specific IgA in plasma by means of an ELISA assay. During immunization IgA antibodies against SIV gp130 and SIV capsid protein were transiently detectable at low titers in all animals from the mucosal group and in five of six monkeys from the systemic group (Table 1). Following challenge all vaccinees showed a rapid surge in their SIV IgA antibodies by week 2 which was significantly higher in the mucosal group compared to the systemic one ($p < 0.05$). In rectal swabs we did not detect SIV-specific IgA (data not shown). Either these responses were not induced at all or their absence could have been due to technical problems concerning sample storage.

Adenovirus neutralization transiently affected SIV-specific T-cell proliferation

Neutralizing antibody titers against adenovirus (nAbrAd5) were measured by a green fluorescent foci reduction assay. They were

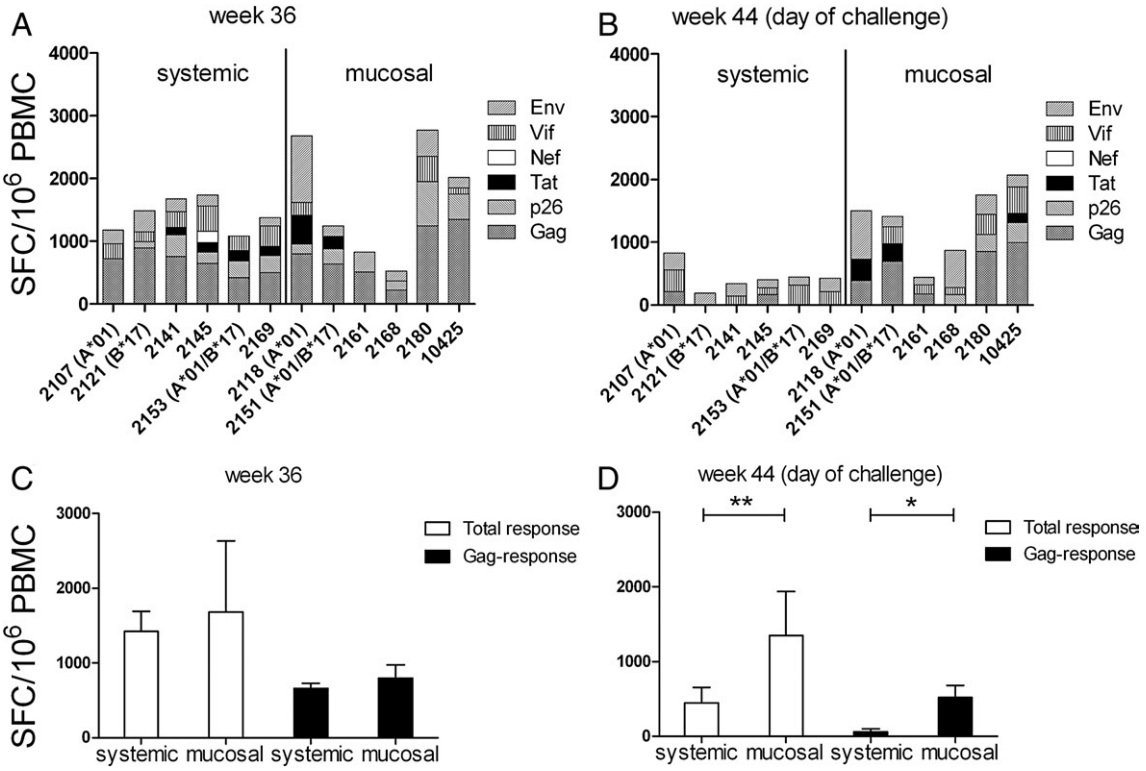

Fig. 2. Individual cumulative IFN-γ ELISpot responses against different SIV peptide pools in the two vaccine groups at week 36 (4 weeks after final immunization) (A) and at week 44 (day of challenge) (B) and the mean of the total and the Gag ELISpot responses for each group at week 36 (C) and at week 44 (D) are shown. The Gag- and the total ELISpot responses were significantly higher in the mucosal group on the day of challenge but not at 4 weeks after final immunization. Systemic, vaccine group systemically immunized; mucosal, vaccine group where systemic and mucosal vaccine application were combined; A*01 indicates the *Mamu-A*01*, B*17 the *Mamu-B*17* animals.*, p-value<0.05; **, p-value<0.01.

elevated 2 weeks after the first systemic rAd5 immunization in the systemic group; while no titers were scored in the mucosal group after oral rAd5 application (Fig. 3B). Four weeks after the second rAd5 application, peak nAbrAd5 titers were reached in the systemic group, and these were significantly higher than those in the mucosal group. The difference in vector-specific immunity between the two vaccine groups had dissipated by the time of challenge.

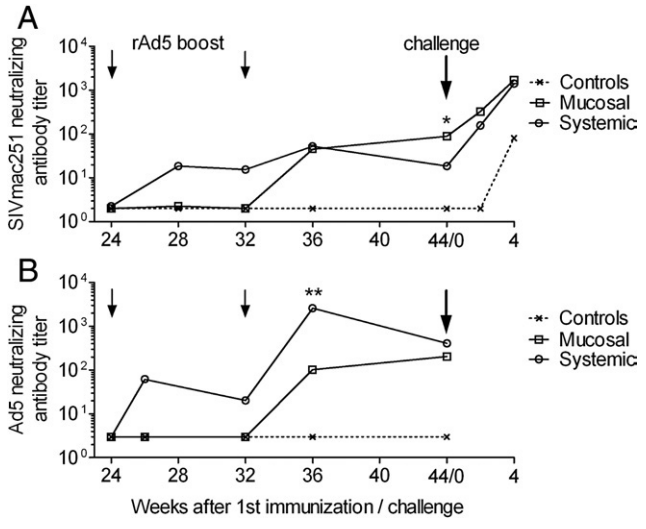

Fig. 3. Geometric means of neutralizing antibodies against SIVmac251/32H during immunization and after challenge measured by sMAGI-assay (A) and against adenovirus measured in a green fluorescent foci reduction assay (B). Values are shown as reciprocals of serum dilution yielding >80% (SIV) and >50% (adenovirus) reduction, respectively of focus forming units. *, significant (p<0.05) and ** highly significant (p<0. 01) differences between the systemic group compared to the mucosal group. The arrows indicate the immunizations and day of challenge.

Next we investigated whether these nAbrAd5 titers had any impact on the SIV-specific immune responses. Correlation analysis showed that the pre-challenge nAbrAd5 titers did not significantly negate subsequent pre- or post-challenge INF-γ ELISpot or antibody responses as obvious by the lack of significant association. However, 4 weeks after the second rAd5 immunization, the point in time with the largest difference in nAbrAd5 titers between the groups with respect to vaccine–vector neutralization, the increased levels of nAbrAd5 titers were associated with suppressed T-cell proliferation appearing 4 weeks later at week 40 (p=0.004).

Reduced plasma viral RNA load during acute infection and its association with SIV-neutralizing antibodies

Although the oral mucosa is not considered to be major viral entry sites in heterosexual HIV transmission, we performed an oral challenge. This mucosal route is very reliable compared to experimental rectal infection (own unpublished data, Koopman et al. 2004), truly atraumatic (Stahl-Hennig et al. 1999) and a single exposure requires less infectious units (6000 $TCID_{50}$) compared to vaginal infection (10^5 $TCID_{50}$; Miller et al. 2005).

After oral SIVmac239 challenge at week 44, plasma viral load was quantified by real time PCR. Plasma viremia is reported as geometric means for each group (Fig. 4A). All animals became productively infected at 2 wpc (Figs. 4B–D). However, vaccinated macaques demonstrated significant control of acute viremia 2 and 4 wpc (p=0.002) independent of the route of immunization. This reduction in viremia was maintained only shortly after the acute phase, and did not last beyond 12 to16 wpc (Fig. 4A). The lack of significant differ-

Table 1
SIVgp130 and -p27 specific IgA-titer in serum of vaccinees during immunization and after challenge

wpi/wpc	Mucosal group						Systemic group					
	2118	2151	2161	2168	2180	10425	2107	2121	2141	2145	2153	2169
SIVgp130												
24	5	10	<5	<5	<5	<5	<5	<5	<5	<5	<5	10
32	5	5	<5	<5	<5	5	<5	<5	5	<5	<5	10
34	80	80	5	<5	40	10	10	10	20	<5	10	40
44/0	20	10	<5	5	5	<5	<5	<5	5	<5	<5	40
46/2	80	160	160	80	320	80	20	80	40	20	10	160
48/4	160	160	80	160	80	40	10	80	40	40	40	160
SIVp27												
24	10	5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
32	20	<5	<5	10	<5	<5	<5	<5	5	<5	<5	10
34	40	5	5	5	5	<5	5	10	20	<5	5	40
44/0	5	<5	<5	20	10	5	5	<5	5	<5	<5	20
46/2	40	80	160	320	320	320	160	80	640	80	20	640
48/4	40	80	160	160	160	160	160	160	320	20	20	1280

wpi: weeks after first immunization; wpc: weeks after challenge.
The four- or five-digit numbers are macaque designations.

ences between the controls and vaccinees might be partly explained by the early death of two rapid progressors in the control group (Fig. 4B and Table 2).

In order to assess the impact of neutralizing SIV antibodies on viremia, these were correlated with plasma viral load. The titer of nAbs at 2 wpc correlated inversely with the RNA load at 2 and 4 wpc ($p \leq 0.03$) indicating a role of nAb responses to SIV in control of viral replication during acute infection.

Better conservation of CD4$^+$ memory cells in vaccinees after challenge

As a measure of vaccine efficacy and to follow the clinical and immunological progress of the animals, T-cells were analyzed by multicolor flow cytometry at regular intervals. To monitor changes in memory cells in the different study arms, T-cell memory subsets were measured and normalized to pre-infection values. We considered the analysis of CD4$^+$195$^+$ T-cells to be important since these are usually the first targets of SIV-infection (Mattapallil et al., 2005). This population was significantly conserved in the vaccinees within the first 4 weeks after infection while it was largely reduced in the controls (data not shown). CD4$^+$ central-memory cells (TCM) defined as CD4$^+$29$^+$95$^+$28$^+$ T-cells, were significantly reduced in control animals compared to vaccinees during the acute phase at 2 wpc (Fig. 5). Additional analyses of CD8$^+$ memory T-cells revealed a strong and short-lived increase of central-memory subsets at 2 wpc in the vaccinees. By contrast, only the proportion of effector-memory cells (TEM) increased in controls at this time point. We observed no differences in the naïve T-cell subset between control animals and vaccinees (data not shown).

Preservation of CD4$^+$ memory T-cells correlated with SIV specific T- and B-cell responses and viral load

Because of the pronounced differences in the degree of TCM preservation at 2 wpc, we used this point in time to compute correlation statistics. The proliferation responses occurring 4 weeks after second Ad5 immunization until 2 weeks after challenge were found to consistently correlate positively with TCM values. Moreover, the proportion of these cells during the acute infection phase inversely correlated with the plasma viral RNA load from 2 to 20 weeks post-challenge ($p < 0.03$), coinciding with significantly lower viral loads in both vaccine groups. The proportions of these TCM cells also correlated with higher titers of SIV binding antibodies to p27 and gp130 during the acute and post-acute phase of infection ($p < 0.04$). These data indicate a strong anamnestic immune response linked to T-helper cells, particularly due to conserved TCM.

Mhc class I genotypes control latent but not acute viremia

Rhesus macaques were *Mhc*-typed to assign those carrying similar *Mhc*-class I genotypes evenly to the three experimental groups (Table 2). We had demonstrated earlier that vaccinees controlled acute viremia better than control animals (Figs. 4A–D). Remarkably, this viremia control between vaccinees and control animals at 2 wpc was still evident even after excluding those immunized macaques that were positive for the *Mamu-A*01* allele in our analyses ($p = 0.004$) and remained so until 8 wpc ($p < 0.05$) (data not shown). Moreover, there was no difference in viremia control between *Mamu-A*01* positive and negative vaccinated animals during the acute phase of infection (Fig. 4E). During this period, the genetic background did not seem to exert direct influence on acute viremia control. But at later time-points starting 12 wpc, *Mamu-A*01* positive vaccinees controlled viral replication more efficiently than the other immunized macaques that were *Mamu-A*01* negative ($p < 0.04$, Fig. 4E). To allow for a more detailed assessment of the effects of vaccination, four *Mhc*-identical half-sibs positive for *Mamu-A1*01* and *-B*17* were included in this study; two of them being immunized, the other two serving as controls. All of them became elite controllers after SIV infection. Although a statistical analysis is not possible with these small numbers, there is a trend that the two immunized macaques reduced peak viremia and were able to control viral replication below detection limits earlier than the two non-vaccinated macaques (Fig. 4F). Of two *Mhc*-identical half-sibs displaying rapid disease progression, the mucosally immunized macaque 2180 survived nearly twice as long as the non-immunized macaque 2175 (27 vs. 14 wpc, Table 2). Thus, in these macaques a positive effect of the vaccination was evident.

For further comparison four *Mhc*-similar macaques carrying a genotype associated with slow disease progression (Sauermann et al., 2008) were included. Again statistical analysis was not possible due to small group sizes, but compared to the non-vaccinated macaques (2165 and 2194), the two immunized ones (2168 and 2169) had a reduced viral load at peak viremia. The individual viral set point of these four animals was reached after 24 weeks of infection (Fig. 4G). Unfortunately, one immunized macaque (2169) had to be euthanized after 27 wpc due to an SIV-unrelated disease (Table 2). The other macaque (2168) that was immunized mucosally survived longer than the two control macaques.

To compare the effects of immunization, two other *Mhc*-similar macaques, 2161 and 2145 carrying the *Mhc*-alleles *Mamu-B*65* and *-B*6901* were included in the vaccine groups. The mucosally immunized macaque 2161 also outlasted the systemically immunized animal 2145 for 6 months (Table 2). However, Kaplan–Meier analysis on sur-

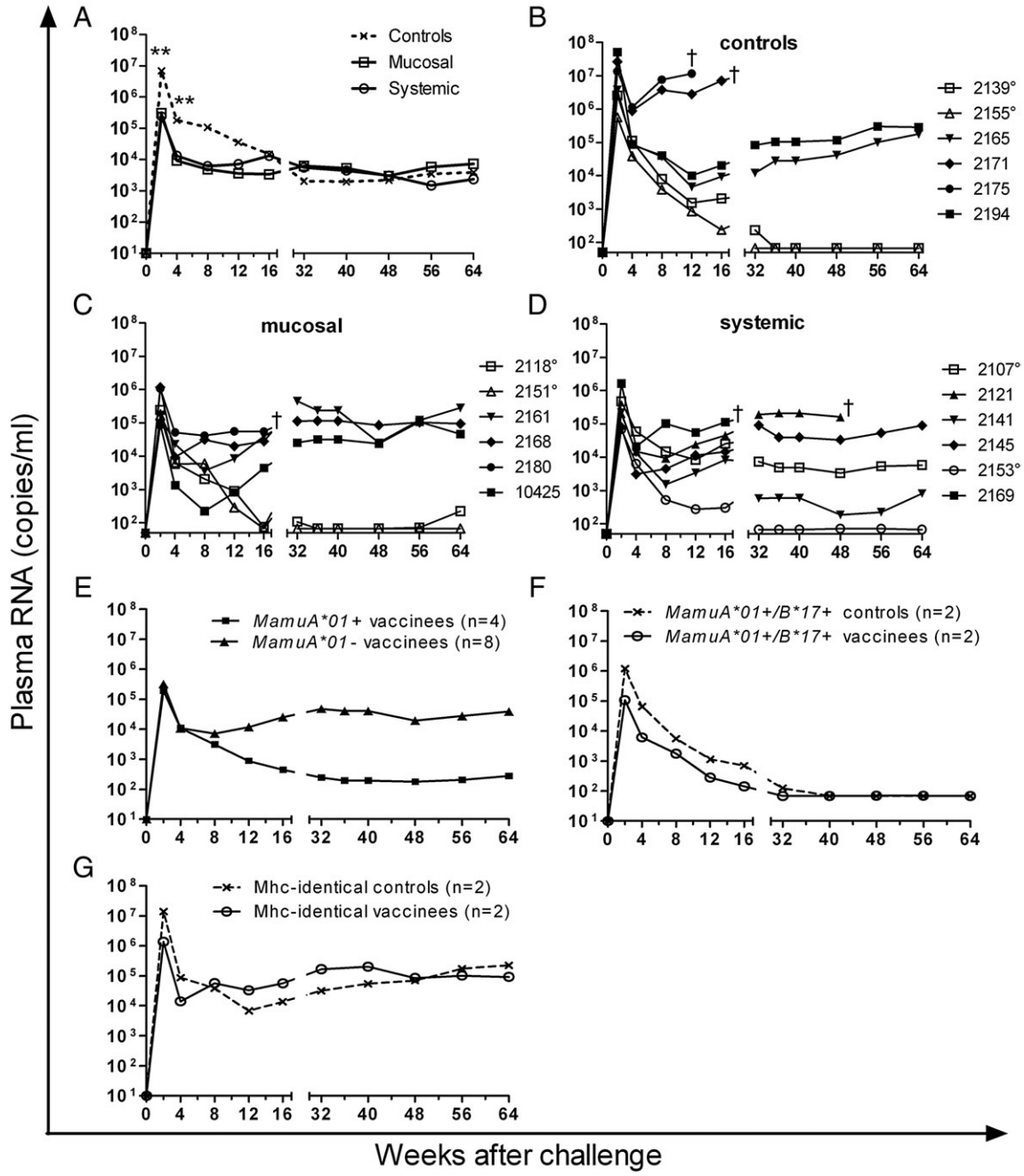

Fig. 4. Viral loads in vaccinated and control animals and influence of *Mhc* class 1 allele on viremia control after oral challenge with SIVmac239. RNA viral loads expressed as RNA equivalents per milliliter plasma are shown as geometric mean of each group (A), for the individual animals per group (B–D), for *Mamu-A*01* positive, respectively negative vaccinees (E), for *Mamu-A*01/B*17 Mhc* identical animals (F) and for the four *Mhc*-identical siblings (G). **, *p*-value<0.01; †, death; °, indicates the *Mamu-A*01*animals.

vival of the two vaccine groups and the controls reached no statistical significance.

In conclusion, the combined data of the different experimental groups as well as the single observations of *Mhc*-identical macaques show that the significant reduction in acute viremia was induced by vaccination and that genetic background played a major role late after resolution of acute infection. In addition, including mucosal immunization appeared to prolong the survival time in our study animals (Table 2).

Discussion

In pre-clinical AIDS vaccine studies utilizing the non-human primate model DNA prime–viral vector booster immunization strategies in which vaccines were given systemically have been widely pursued (Casimiro et al., 2005; De Rose et al., 2007; Hanke et al., 1999; Hel et al., 2002; Letvin et al., 2006; Makitalo et al., 2004; Mattapallil et al., 2005; Shiver et al., 2002; Stolte-Leeb et al., 2008; Suh et al., 2006).

In our study we applied different immunization routes using a SIV-DNA/recombinant adenovirus prime boost regimen to investigate both humoral and T-cell immune responses and to analyze for efficacy. We demonstrated in DNA vaccine-primed rhesus macaques that consecutive oral and systemic booster immunizations with rAd5 induced a stronger Gag-specific T-cell, lymphoproliferative as well as binding and neutralization antibody response against SIV than two corresponding systemic immunizations. However, there were no differences between the vaccine groups in vaccine-induced SIV-specific IgA responses, the

Table 2
Mhc class I alleles of the experimental macaques

Animal[a]	*MhcMamu-A*-alleles	*MhcMamu-B*-alleles	Survival (weeks post-challenge)
Sham vaccinated macaques			
2139[b]	*A1*01, A2*05 A1*08, A3*1303, A2*05*	*B*17, B*29 B*21, B*28, B*45*	Alive 112
2155[b]	*A1*01, A2*05 A1*08, A3*1303, A2*05*	*B*17, B*29 B*21, B*28, B*45*	Alive 112
2171	*A1*04, A4*1403*	*B*12, B*22, B*3001, B*46, B*49, B*57*	25
2165[c]	*A1*08, A3*1303, A2*05*	*B*38, B*46, B*4701*	102
	*A1*04, A4*1403*	*B*12, B*22, B*3001, B*46, B*49, B*57*	
2175[d,e]	*A1*11, A2*05*	*B*48*	14
	*A1*04, A4*1403*	*B*12, B*22, B*3001, B*46, B*49, B*57*	
2194[c]	*A1*08, A3*1303, A2*05*	*B*38, B*46, B*4701*	110
	*A1*04, A4*1403*	*B*12, B*22, B*3001, B*46, B*49, B*57*	
Systemic group			
2107	*A1*01, A2*05 A1*08, A3*1303, A2*05*	*B*22, B*12 B*26, B*27, B*3003, B*43, B*49, B*57*	Alive 112
2121	*A2*05, A4*1403 A1*02, A3*1302*	*B*17, B*2901 B*01, B*07, B*3002, B*57*	48
2141[d]	*A1*04, A4*1403 A1*08, A3*1303, A2*05*	*B*38, B*48, B*41 B*21, B*28, B*45*	Alive 112
2145	*A1*08, A3*1303, A2*05*	*B*65, B*6901, B*46*	77
	*A1*08, A3*1303, A2*05*	*B*21, B*28, B*45*	
2153[b]	*A1*01, A2*05 A1*08, A3*1303, A2*05*	*B*17, B*29 B*21, B*28, B*45*	Alive 112
2169[c]	*A1*08, A3*1303, A2*05*	*B*38, B*46, B*4701*	27
	*A1*04, A4*1403*	*B*12, B*22, B*3001, B*46, B*49, B*57*	
Mucosal group			
2118[d]	*A1*01, A2*05 A1*02, A3*1302*	*B*22, B*12 B*01, B*07, B*3002, B*57*	Alive 112
2151[b]	*A1*01, A2*05 A1*08, A3*1303, A2*05*	*B*17, B*29 B*21, B*28, B*45*	Alive 112
2161	*A1*04, A4*1403*	*B*65, B*6901, B*46 B*38, B*12, B*3001, B*46, B*49, B*57*	101
2168[c]	*A1*08, A3*1303, A2*05*	*B*38, B*46, B*4701*	Alive 112
	*A1*04, A4*1403*	*B*12, B*22, B*3001, B*46, B*49, B*57*	
2180[d,e]	*A1*11, A2*05*	*B*48*	27
	*A1*04, A4*1403*	*B*12, B*22, B*3001, B*49, B*57*	
10425[d]	*A1*04, A4*1403*	*B*69, B*48, B*41*	Alive 112

[a] The four or five-digit numbers are macaque designations.
[b] The macaques represent *Mhc*-identical siblings.
[c] The macaques have identical *Mhc* class 1 alleles.
[d] The macaque carries more *Mhc* class 1 genes judged by the DNA-sequences from its parents.
[e] The macaques represent *Mhc*-identical siblings.

levels of circulating CD4+ memory T-cells in blood and viral loads in the acute phase of infection.

When the first rAd5 booster immunization was given orally by spray followed by a systemic application of the same vectors, IFN-γ-secretion of PBMCs was increased in this group coinciding with a broader immune response on the day of challenge. Such a response is supposed to be important for reduction of viral load (Hel et al., 2006). Maybe the inclusion of IL-15 vectors caused this stronger response of the mucosal group as it has been shown in mice that mucosal application of IL-15-expressing vectors enhanced the generation of CD8+ effector T-cells (Toka and Rouse, 2005). In addition, the immunostimulatory peptide is known to activate immature and mature dendritic cells (Yang et al., 2002) which reside at high numbers in mucosal tissues. This activation may have led to an increased immune response in the mucosal group. Moreover, inclusion of a mucosal vector application led to a strong T-cell proliferative response on the day of challenge which is known to contribute to a favorable long-term virological outcome (Michelini et al., 2004; Spring et al., 2001). Additionally, this T-cell proliferation significantly correlated with neutralizing and binding SIV-antibodies determined on the same day and 2 weeks thereafter. This emphasizes the supportive role of T-cell proliferation for generating B-cell responses thus probably contributing to a favorable disease course.

The nAb titers against SIVmac251 reached a plateau following the first systemic rAd5 boosting in the systemic group. By comparison, mucosal immunization alone did not generate any measurable nAb titers, possibly due to slow or transient mucosal uptake of the vector antigen, but the nAbs appeared after systemic boosting. In our study, a combination of mucosal and systemic immunization secured significantly higher SIV-nAb responses compared to a systemic only regimen as reported previously (Barnett et al., 2008). Moreover, in our vaccinees reduced levels of acute viremia correlated inversely with the titer of SIV-nAb at 2 wpc, confirming the necessity to mount this kind of anamnestic response for early viral containment. Limited analysis of humoral responses considered to be linked to mucosal compartments (i.e. IgA in blood) revealed the presence of SIV-specific IgA in some animals after final immunization and an anamnestic response in all vaccinees independent of the route of immunization. On the basis of all of these results we hypothesize that oral rAd5 administration potently primes for greater and longer lasting subsequent immunological responses.

The mechanism by which oral immunization followed by intramuscular boost with rAd5 vaccine elicited the enhanced T-cell and antibody responses remains to be elucidated. However, several reports suggested potential advantages of mucosal vaccination when given prior to systemic immunization (Ciabattini et al., 2004; Huang et al., 2007a, 2007b) since mucosal immunization induces both mucosal and systemic immune responses (Makitalo et al., 2004) while intramuscular immunization preferentially stimulates systemic responses. In future experiments, more sophisticated methods need to be applied to analyze for immune responses in different mucosal compartments for which particularly the vagina and intestine are regarded as the first site of defense against HIV/SIV entry (Mattapallil et al., 2005).

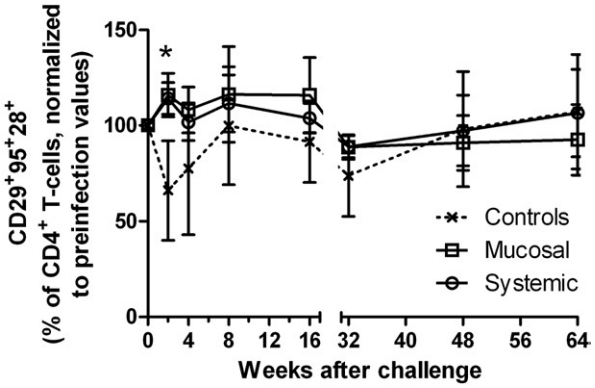

Fig. 5. Proportion of central-memory T-cells (TCM) in vaccinated and control animals after oral challenge with SIVmac239. TCM were defined as $CD4^{+}29^{+}95^{+}28^{+}$ and expressed as percent normalized to pre-infection values. Standard deviations are indicated by vertical bars. *, p-value<0.05.

We have previously reported that the loss of $CD4^+$ T-cells was delayed in vaccinated macaques able to control acute viremia (Suh et al., 2006). Additional literature supports the relevance of memory T-cell preservation on vaccine outcome (Letvin et al., 2006; Mattapallil et al., 2006). Here we investigated whether preservation of T-cell memory was related to viremia control. Compared to all vaccinees, the population of TCM significantly decreased in control animals at 2 wpc. In all animals the level of this T-cell subset inversely correlated with plasma viremia during a period spanning 20 weeks after infection and also correlated with SIV binding antibodies during acute infection indicating the impact of the memory T-cell subset on viremia control and SIV-specific antibody production. These particular memory T-cells have been described previously to be associated with better clinical outcome (Letvin et al., 2006). We further assessed whether the T-cell proliferation was associated with preservation of this critical $CD4^+$ T-cell population. Lymphoproliferative responses on the day of challenge were positively and significantly associated with maintained levels of $CD4^+$ memory T-cells at peak viremia underlining the importance of this functional immune response in suppressing pathogenic sequelae of infection.

It is not clear why despite a stronger systemic T- and B-cell immunity in the mucosal group the level of viremia control was similar in both vaccine groups. Maybe even our mucosal vaccine strategy did not induce immune response at relevant sites like the gut where massive viral replication occurs early on. In addition to the adaptive responses determined, other factors such as innate immunity may play a role in acute viremia control in SIV-infected macaques (Giavedoni et al., 2000) and in the preservation of $CD4^+$ memory T-cells. However, activation of the innate immunity by the vector alone can be ruled out since we have demonstrated earlier that application of Ad5 expressing an SIV-unrelated gene did not influence viral load after SIV-challenge (Suh et al., 2006).

Moreover, we analyzed for NK activity early after challenge by incubating PBMC with NK-sensitive K562 cells. Despite no statistical significance, a lower activity of NK-cells was observed in the vaccinees compared to the controls (data not shown) suggesting that the adaptive immune response had a more profound effect on viremia control.

Due to the failed human phase IIB STEP vaccine trial there is increasing concern about reduced efficacy of adenoviral vaccine vectors occasioned by elicitation of anti-adenoviral antibodies (Casimiro et al., 2005; Sekaly, 2008). Consequently, we determined the presence of these antibodies during immunization and their influence on immunization and infection outcome. We confirmed that a single mucosal rAd5 application alone did not raise any measurable nAbAd5 titers (Stahl-Hennig et al., 2007a, 2007b). Only after a systemic application nAb against Ad5 levels rose in both vaccine groups. In general, nAb against Ad5 are considered to mitigate immune responses against the incorporated genes. In our case, such vector-specific immunity might have inhibited the burst of immune reactions in the systemic group as the response at week 36 was inversely correlated with lymphocyte proliferation at week 40 ($p<0.05$). The increased production of neutralization antibodies to rAd5 (Fig. 3B) most likely interfered with boosting of helper T-cell responses in the systemic group, resulting in weaker antibody and IFN-γ ELISPOT T-cell responses.

In contrast to the STEP study we used a heterologous prime-boost regimen with a more attenuated Ad5. This setting secured a broad CTL-response (Fig. 2) required for an effective HIV-vaccine (Watkins et al., 2008). Although the differences in immune responses between the mucosal and systemic group were not reflected in viremia control, the data clearly show that the combination of mucosal and systemic immunization provides an alternative avenue to repetitive invasive procedures characterizing systemic immunization.

Since vaccinated animals controlled acute viremia significantly better than unvaccinated ones independent of the immunization route, we aimed to assess whether this viremia control was also influenced by the *Mhc*-class 1 background. We demonstrated that vaccination protected from initial high viral loads independent of the test animals' genetic composition as shown before (Letvin et al., 2006). Our vaccine-mediated reduction of viral load lasted until 8 wpc. Thereafter, the animals' genetic background significantly affected viremia control. This effect was further corroborated by the inclusion of *Mhc*-identical siblings, confirming an attenuation of the vaccine effect during the post-acute and chronic phases of infection and the dominance of *Mhc*-driven influence on viremia control.

Taken together, our vaccination strategy procured strong SIV Gag-specific IFN-γ and T-cell proliferation responses in both vaccine groups which were independent of *Mhc*-background. Broader and stronger SIV-specific and reduced vector-specific immune reactions were induced in the mucosally immunized animals. Despite this increased immunogenicity there was no significant difference in the vaccine efficacy between the two vaccine arms. Nevertheless, these findings strongly argue for the inclusion of mucosal vector administration in any AIDS-vaccine approach as it is less invasive, easily applicable and could reduce acceptability issues.

Materials and methods

Animals

Eighteen rhesus macaques of Indian origin, seronegative for SIV, simian retrovirus and T-cell leukemia virus, were housed at the German Primate Center under standard conditions according to the German animal protection law which complies with the European Union guidelines on the use of non-human primates for biomedical research. Major histocompatibility complex (*Mhc*) class I allele genotyping of the macaques was done as described elsewhere (Muehl et al., 2002; Sauermann et al., 2008). The animals were assigned to three experimental groups of six animals each (two vaccine groups and one control group). We included four *Mhc* identical siblings which were positive for both *Mamu-A1*01* and *-B*17*. Two of those were assigned to the control group and one each to the vaccine groups. To balance the number of *Mamu-A1*01+* animals in the study groups, one additional *Mamu-A1*01+ B*17-* animal was allocated to each of the two vaccine groups. Every 2 to 4 weeks during immunization and post-challenge (wpc), blood specimens were collected by venipuncture. Physical examination, bleeding and immunizations were carried out under ketamine anesthesia.

Vaccination and challenge

A vaccine regimen comprising DNA priming and rAd5 boosting was employed. The DNA regimen included four different pGX10 plasmid vectors expressing a codon-optimized SIV *gag*, SIV*envΔtransmembrane*, SIV*nef-tat-vpx-vif-tat* or human IL-15 (1.5 mg/construct). Since Tat-specific responses have been associated with slow AIDS progression in HIV-infected humans (van Baalen et al., 1997, Zagury et al., 1998) the *tat* gene was included twice into the construct in order to present larger amounts of Tat peptides to the immune system. The amount of Nef-Tat-Vpx-Vif-Tat protein in DNA-transfected cells was verified by Western blot using anti-SIV Nef antibodies (ARP4007, NIBSC). It was comparable to that of Nef protein expressed by transfection of *nef* DNA alone, indicating that fusion of the regulatory genes did not affect expression of the protein. We also included 1 mg of an immunostimulatory peptide (Trp-Lys-Tyr-Met-Val-$_D$-Met; Yang et al., 2002) in each DNA application. For booster immunizations five E1/E3 deleted replication-incompetent rAd5 vectors were used expressing SIV *gag*, *env* or human IL-15 (rAd5-IL-15) as described above and non-codon optimized *vif-nef* or *tat-vpx* as reported (Suh et al., 2006). The rAd5 vectors expressing the different SIV genes are later referred to as rAd5-SIV. The original SIV envelope transmembrane sequence was truncated and the signal sequence of the *env* gene was replaced by a tissue plasminogen activator signal sequence in order to enhance secretion of

soluble envelope. The sham vaccine regimen consisted of sodium phosphate buffer for DNA and Tris buffer for rAd5.

The two vaccine groups received an intramuscular (i.m.) DNA priming at weeks 0, 8 and 16. After priming, one group was boosted twice i.m. (systemic group) with escalating doses of rAd5-SIV at weeks 24 (1×10^8 pfu/construct) and 32 (2×10^9 pfu/construct) while the other group received the same boost first mucosally (mucosal group) via oropharyngeal spray (40) (1×10^9 pfu/construct) and then i.m. (2×10^9 pfu/construct) at the respective time points. The control group received the respective buffers i.m. at the corresponding time points. Twelve weeks after final immunization all animals were challenged orally with approximately 6000 median tissue culture infectious doses of SIVmac239 as described elsewhere (Kuate et al., 2006).

IFN-γ ELISpot and lymphoproliferation assay

IFN-γ ELISpot assay was performed using SIV Gag (EVA7066, NIBSC, UK); Env1 (Cat# 6528–6582, NIH, Maryland, USA) and Env2 (Cat# 6583–6637, NIH), Nef (EVA777, NIBSC) and Tat (EVA7069, NIBSC) peptides as described previously (Suh et al., 2006). In addition, we also used peptide pools comprising p26 (ARP714.1-22, NIBSC) and vif (6205, NIH). The IFN-γ positive cells were counted using a Bioreader®-3000 (Bio-Sys GmbH, Karben, Germany). Individual values obtained by peptide stimulation minus medium control were normalized to individual pre-immunization values and considered positive if yielding a difference greater than 100 spot forming cells (SFC) per million peripheral blood mononuclear cells (PBMC). The arbitrary cut-off was defined to consider individual assay to assay variations. The results from separate stimulations with the Env1 and Env2-peptide pools are presented as the sum of the spot numbers of both pools. A T-cell proliferation assay was performed in triplicate by stimulating fresh PBMCs with aldrithiol-2 treated (AT-2) SIV or inactivated microvesicles derived from SUP-T1 cells (final protein concentration, 1 μg/ml) (Spring et al., 2001). Plates were read by a β-plate reader. The stimulation index (SI) was calculated by dividing the counts per minute (cpm) of the stimuli tested by the cpm of the medium response for each animal's sample. A SI above two was considered as a positive response.

FACS analyses of T-cells

Whole blood lymphocytes were stained with a pre-titrated antibody mixture comprising anti-CD3-Alexa700 (clone SP34-2), CD4-Alexa405 (clone SK3), CD8-AmCyan (clone SK1), CD95-APC (clone DX2), CD28-PerCP-Cy5.5 (clone L293), CD29-FITC (clone 4B4), CD195-PE (clone 3A9) and CD45RA-ECD (clone 2H4). Stained lymphocytes were analyzed for the expression of cell surface markers by flow cytometry on a LSR II flow cytometer (Becton Dickinson, Heidelberg, Germany). Lymphocyte populations were gated based on forward and side scatter characteristics and expression of CD3 surface antigen. Data were acquired with BD FACS Diva 5.1 Software (Becton Dickinson) before analysis with FlowJo 6.4 Software (TreeStar).

Detection of antibody responses

A standard ELISA to detect antibodies against SIV (Kuate et al., 2006) was performed on plates coated with 30 ng per well of recombinant p27 (EVA643, NIBSC) or recombinant SIVgp130 (EVA670, NIBSC). For IgG determination test plasma was diluted 1:200 and the optical densities were measured at 450 nm. SIV-specific IgA antibodies in serum and from rectal swabs were determined with the following modifications. Serum samples were diluted serially without any further adjustments in their immunoglobulin concentrations and reacted with recombinant SIV p27 antigen or gp130 at a concentration of 50 ng per well each. Upon development of a strong colour in the positive control, the staining reaction was stopped and the optical density was measured. The titers were expressed as the reciprocal of the highest dilution yielding optical densities twice above the autologous pre-immunization values.

Neutralizing antibodies against SIVmac were determined with a SIVmac251/32H neutralization assay. Except for a few modifications, the assay was conducted as previously described (Chackerian et al., 1995). Briefly, rhesus macaque mammary tumor cells expressing CD4 and β-galactosidase (sMAGI cell-line, NIH #2955) were seeded into a 96-well U-bottom plate at a concentration of 7000 cells/well in DMEM. An aliquot of SIVmac251/32H generating around 200 infectious foci was incubated for 1 h at 37 °C with ten 3-fold serial dilutions starting from 1:12. Respective serum sample/virus mixtures were added to sMAGI cells and incubated at 37 °C and 5% CO_2 for 50 min before adding 15 μg/ml DEAE dextran. After two days of incubation, plates were fixed and the infectious foci stained in the presence of X-Gal. The neutralizing titer was defined as the reciprocal of the final sample dilution giving more than 80% reduction in infectious foci.

The presence of adenovirus-neutralizing antibodies compromises target-specific immune responses in vaccine regimens that employ adenovirus as a vaccine vector (McConnell et al., 2007; Nwanegbo et al., 2004). Because of this, adenovirus neutralization was evaluated in a green fluorescent foci reduction assay as previously described (Farina et al., 2001). Briefly, complement-inactivated macaque sera were tested in 2-fold serial dilutions starting with a 1:10 dilution. One hundred microliters of diluted sera were added to an equal volume of rAd5 expressing enhanced green fluorescent protein (EGFP) and incubated at 4 °C for 2 h. After incubation, 150 μl of the virus/serum mixture was transferred to corresponding wells of a 96-well flat-bottomed plate pre-plated with 2×10^4 of HEK293 cells. After incubation at 37 °C in 5% CO_2 for 48 h, plates were examined under a fluorescent microscope to determine the percentage of cells expressing EGFP. Neutralizing titers were defined as the reciprocal of the serum sample dilution giving >50% reduction in the number of EGFP-expressing cells compared to infected controls incubated with autologous pre-immunization sera only.

Plasma viral RNA quantitation

Viral RNA was isolated from frozen plasma samples following the MagAttract Virus Mini M48 protocol (Qiagen, Hilden, Germany). Purified SIV RNA was quantified using TaqMan-based real-time PCR on an ABI-Prism 7500 sequence detection system (Applied Biosystems) as described (Negri et al., 2004). Amplified viral RNA was expressed as SIV-RNA copies per milliliter plasma.

Statistics

Data on the number of RNA copies, ELISA titers, proliferation assays, neutralization titers, survival time and T-cells were analyzed with GraphPad Prism version 4 (GraphPad Software). Comparisons between groups were performed with the nonparametric two tailed Mann–Whitney's *U* test. Associations between parameters were analyzed with two tailed Pearson's correlation. Differences and associations between groups were considered significant at *p*-values less than 0.05.

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