Host factors determine differential disease progression after infection with nef-deleted simian immunodeficiency virus

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Infection of macaques with live attenuated simian immunodeficiency virus (SIV) usually results in long-lasting efficient protection against infection with pathogenic immunodeficiency viruses. However, attenuation by deletion of regulatory genes such as nef is not complete, leading to a high viral load and fatal disease in some animals. To characterize immunological parameters and polymorphic host factors, we studied 17 rhesus macaques infected with attenuated SIVmac239ΔNU. Eight animals were able to control viral replication, whereas the remaining animals (non-controllers) displayed variable set-point viral loads. Peak viral load at 2 weeks post-infection (p.i.) correlated significantly with set-point viral load (P<0.0001). CD4+ T-cell frequencies differed significantly soon after infection between controllers and non-controllers. Abnormal B-cell activation previously ascribed to Nef function could already be observed in non-controllers 8 weeks after infection despite the absence of Nef. Two non-controllers developed an AIDS-like disease within 102 weeks p.i. Virus from these animals transmitted to naïve animals replicated at low levels and the recipients did not develop immunodeficiency. This suggested that host factors determined differential viral load and subsequent disease course. Known Mhc class I alleles associated with disease progression in SIV WT infection only marginally influenced the viral load in Δnef-infected animals. Protection from SIVmac251 was associated with homozygosity for MHC class II in conjunction with a TLR7 polymorphism and showed a trend with initial viral replication. We speculated that host factors whose effects were usually masked by Nef were responsible for the different disease courses in individual animals upon infection with nef-deleted viruses.

INTRODUCTION

Infection or immunization with live attenuated human immunodeficiency virus (HIV) or simian immunodeficiency virus (SIV) protects efficiently against symptoms of AIDS or infection with pathogenic immunodeficiency viruses (Daniel et al., 1992; Gorry et al., 2007; Koff et al., 2006; Whitney & Ruprecht, 2004). The commonly used live attenuated viruses for experimental immunization of macaques harbour deletions in the viral nef gene. Vaccination with live attenuated viruses is not only very potent in terms of protection against challenge with pathogenic virus, but also with respect to its durability. As early as 3 weeks and as late as 2 years after immunization, juvenile and adult macaques can be protected against superinfection with pathogenic SIV (Sharpe et al., 2004; Stebbings et al., 2004). The immune mechanisms conferring protection have proven to be difficult to identify. Several reports have shown that protection can be obtained in the presence of low or even absent cellular or humoral immune responses (Mansfield et al., 2008; Nilsson et al., 1998; Stahl-Hennig et al., 2007; Stebbings et al., 2005).

The idea to immunize humans against HIV with live attenuated viruses has been dismissed because many if not two supplementary tables are available with the online version of this paper.
all deletion constructs can ultimately cause disease in infected infants and, after prolonged observation, also in adult monkeys (Baba et al., 1999; Chakrabarti et al., 2003; Connor et al., 1998; Hofmann-Lehmann et al., 2003; Sawai et al., 2000). Progression to immunodeficiency has also been observed in humans infected by blood transfusion from a HIV-1-infected donor carrying a deletion in nef (Gorry et al., 2007). In addition, superinfection with pathogenic SIV can lead to recombination events and thus to the generation of novel pathogenic viruses (Gundlach et al., 2000; Reynolds et al., 2008).

Viral load is generally lower in monkeys infected with SIVmacΔnef compared with WT SIV-infected macaques, but large individual differences can be observed, especially in rhesus macaques of Indian origin. Many factors may contribute to these differences and consequently to the virulence of attenuated SIV. The unique, not fully developed neonatal immune system is clearly a risk factor. Furthermore, partial refilling of the nef deletion leading to the production of a truncated viral Nef protein as well as a series of other alterations in the viral genome may increase the virulence of the virus (Alexander et al., 2003; Sawai et al., 2000; Whatmore et al., 1995). In addition, host factors may determine differential viral replication and disease manifestation. Among the host factors influencing disease progression in immunodeficiency virus-infected humans and monkeys, polymorphic genes encoded in the MHC region represent the most important factors (Fellay et al., 2009; Siddiqui et al., 2009). In addition, polymorphisms within the Toll-like receptor 7 (TLR7) have been reported to modulate disease progression in HIV-1-infected humans and SIV-infected macaques (Oh et al., 2009; Siddiqui et al., 2011).

As the factors responsible for differences in disease progression after infection with SIVmacΔnef have not yet been determined fully, the aim of this study was to characterize immunological parameters as well as host factors that contributed to differential disease outcome after experimental infection of rhesus monkeys with the SIVmac239ANU strain that carries a 513 bp deletion in nef (Gundlach et al., 1997). We performed transmission experiments to investigate whether host factors or an enhanced virulence of the virus were responsible for high viral replication in some macaques. The results showed that host factors were important for viral containment. However, they may be different from those influencing pathogenic SIV infection.

**RESULTS**

**Variation of viral load and disease progression in rhesus monkeys infected with nef-deleted SIVmac**

Seventeen rhesus macaques (*Macaca mulatta*) of Indian origin were infected with SIVmac239ANU (Gundlach et al., 1997) either intravenously or via the tonsils. The infection route did not significantly influence viral RNA copies in plasma, at peak viraemia 2 weeks post-infection (p.i.) or at viral set point (24 weeks p.i.) (P = 0.174 and P = 0.81, respectively; Mann-Whitney test). Eleven macaques were observed for at least 64 weeks p.i. The other six macaques were challenged with SIVmac251 at 26 weeks p.i. (Tables 1 and S1, available in the online Supplementary Material) (Tenner-Racz et al., 2004).

In all monkeys, the initial peak viraemia was reduced by one to four orders of magnitude within the first 24 weeks p.i., coinciding with the onset of an immune response (Fig. 1a, Table S1) that resulted in a wide variation of set-point viraemia. Based on the viral load at 24/26 weeks p.i., we stratified the animals in two groups: the controllers, displaying a plasma viral load <300 RNA copies ml⁻¹, and the non-controllers, having a plasma viral load >300 RNA copies ml⁻¹ (Tables 1 and S1). The controllers (n = 8) were inoculated with pathogenic SIVmac251 at 26 or 64 weeks p.i. As expected, they maintained, at least initially, their controller phenotype, indicating that they had developed a vaccine-induced protective anti-SIV immune response (Stahl-Hennig et al., 2007; Tenner-Racz et al., 2004). The non-controllers (n = 9) replicated the virus at varying levels at the viral set point (20–26 weeks p.i.). With the exception of three animals, they were observed for at least 62 weeks p.i. Three macaques (Mm1968, Mm1969 and Mm9037) were challenged with WT SIVmac251 at 26 weeks p.i. They were included as their early viral replication kinetics clearly distinguished them from the controllers (Table 1, Fig. 1). Mm1968 and Mm1969 replicated SIVmac251 at time of death (Freissmuth et al., 2010). Discrimination between SIVmac239ANU and SIVmac251 was not performed for Mm9037.

Plasma viral RNA copies measured at 2 weeks p.i. before onset of adaptive immune responses already distinguished between controllers and non-controllers (P = 0.0055, Mann-Whitney test, Fig. 1b), and correlated significantly with set-point viral load (P < 0.0001, Spearman rank correlation, Fig. 1c).

In contrast to the controllers, non-controllers showed signs of immunological alterations at time of death after a prolonged observation period (Table 2). Two macaques (Mm1891 and Mm1948) that replicated the virus at high levels with a plasma viral load >10⁴ RNA copies ml⁻¹ plasma at set point had to be euthanized with symptoms of AIDS at 100 and 102 weeks p.i., respectively (Tables 1 and 2). They both developed severe thrombocytopenia by 88 weeks p.i. In addition, Mm1948 presented with severe anemia and opportunistic infections in lung and gut. Furthermore, necropsy revealed that both animals had a non-bacterial thrombotic endocarditis often detected in HIV-1-infected patients (Kaul et al., 1991), SIV-associated arteriopathy of the small lung vessels and a severe generalized hyperplasia of the lymphatic organs. Mm1961, which also replicated SIVmac239ANU to high levels, was euthanized at 84 weeks p.i. with signs of generalized lymphatic hyperplasia in various organs, including gut-associated lymphoid tissues. Pneumonia was an additional finding in this animal.
Table 1. Animal ID, route of infection, viral load at set point, observation time, and MHC and TLR7 genotype

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Route of infection*</th>
<th>Plasma viral RNA copies ml⁻¹ at 24/26 weeks p.i.</th>
<th>Survival (weeks p.i.)</th>
<th>Disease course predicted by MHC class I genotype</th>
<th>Homozygous for MHC class II DQB1-DRB haplotype</th>
<th>TLR7 genotype associated with slow disease progression†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controllers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1937</td>
<td>l.v.</td>
<td>Negative</td>
<td>&gt;64‡</td>
<td>Rapid</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>1939</td>
<td>Tonsils</td>
<td>Negative</td>
<td>&gt;26‡</td>
<td>Slow</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>1964</td>
<td>l.v.</td>
<td>Negative</td>
<td>&gt;26‡</td>
<td>Rapid</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>1982</td>
<td>l.v.</td>
<td>Negative</td>
<td>&gt;26‡</td>
<td>Slow</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>8638</td>
<td>Tonsils</td>
<td>Negative</td>
<td>&gt;62</td>
<td>Unknown</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8785</td>
<td>l.v.</td>
<td>Negative</td>
<td>&gt;64‡</td>
<td>Unknown</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>8790</td>
<td>l.v.</td>
<td>Negative</td>
<td>&gt;64‡</td>
<td>Moderate</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>9026</td>
<td>l.v.</td>
<td>220, 32 weeks p.i. Negative</td>
<td>&gt;64‡</td>
<td>Moderate</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Non-controllers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1891</td>
<td>l.v.</td>
<td>29 000</td>
<td>102</td>
<td>Unknown</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>1948</td>
<td>l.v.</td>
<td>54 000</td>
<td>100</td>
<td>Unknown</td>
<td>No</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>1961</td>
<td>Tonsils</td>
<td>7900</td>
<td>84</td>
<td>Unknown</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>1968</td>
<td>Tonsils</td>
<td>10 000</td>
<td>&gt;26‡</td>
<td>Unknown</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>1969</td>
<td>i.v.</td>
<td>3200</td>
<td>&gt;26‡</td>
<td>Unknown</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8637</td>
<td>Tonsils</td>
<td>680</td>
<td>&gt;62‡</td>
<td>Unknown</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>8768</td>
<td>l.v.</td>
<td>560</td>
<td>180</td>
<td>Unknown</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>9037</td>
<td>Tonsils</td>
<td>1400</td>
<td>&gt;26‡</td>
<td>Unknown</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>9038</td>
<td>l.v.</td>
<td>2500</td>
<td>185</td>
<td>Unknown</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Transfused</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1891T</td>
<td>Material from Mm1891; l.v.</td>
<td>1200</td>
<td>58</td>
<td>Unknown</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>1948T</td>
<td>Material from Mm1948; i.v.</td>
<td>1600</td>
<td>61</td>
<td>Moderate</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

* l.v., Intravenous; tonsils, atraumatic via tonsils.
† TLR7 genotype associated with slow disease progression: c.−17C, c.13G (Siddiqui et al., 2011).
‡ Animals were challenged with SIVmac251 at the indicated time point and excluded from further analysis.
Fig. 1. Plasma viral RNA copy numbers (log_{10}) in 17 SIVmac239ΔNU-infected macaques over time. (a) Geometric means of controllers (blue) and non-controllers (red) are indicated by solid lines. Data of individual animals are shown by dots. Plasma RNA copy number of the viraemic controller Mm9038 is shown by a dashed line. Set-point plasma viral load (300 RNA copies ml^{-1}) at 24 weeks p.i. used to distinguish between controllers and non-controllers is indicated by a grey dashed line. (b) Plasma viral RNA copy number 2 weeks p.i. distinguishes controllers from non-controllers (P=0.0055, Mann–Whitney test). (c) RNA copy number at 2 w.p.i. correlates with set-point viral load (P<0.0001, r=0.82, Spearman rank correlation).

Table 2. Findings at time of necropsy of SIVmacΔNU-infected monkeys

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Parasitological results</th>
<th>Microbiological results</th>
<th>Pathohistological findings*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1891</td>
<td>–</td>
<td>–</td>
<td>Non-bacterial thrombotic endocarditis, severe SIV-associated arteriopathy of small lung vessels, severe generalized hyperplasia of LNs, spleen, GALT and BALT</td>
</tr>
<tr>
<td>1961</td>
<td>–</td>
<td>–</td>
<td>Mild generalized lymphatic hyperplasia in several organs (liver, heart, kidney, spleen and GALT), chronic active pneumonia</td>
</tr>
<tr>
<td>8768</td>
<td>–</td>
<td>–</td>
<td>Severe follicular hyperplasia of palatine tonsil and spleen, mild hyperplasia of LNs, moderate interstitial pneumonia, moderate chronic active gastroenteritis</td>
</tr>
<tr>
<td>9038</td>
<td>–</td>
<td>Lung: Pneumocystis jirovecii</td>
<td>Mild generalized hyperplasia of LNs, spleen and GALT, mild chronic active gastroenteritis, active pneumonia</td>
</tr>
<tr>
<td>1891T</td>
<td>–</td>
<td>–</td>
<td>Mild generalized follicular hyperplasia of LNs</td>
</tr>
<tr>
<td>1948T</td>
<td>–</td>
<td>–</td>
<td>Mild generalized hyperplasia of LNs and spleen, mild-to-moderate chronic active gastroenteritis</td>
</tr>
</tbody>
</table>

*LN, lymph node; GALT, gut-associated lymphoid tissue; BALT, bronchial-associated lymphoid tissue.
Loss of CD4+ T-cells, SIV antibody profile and extent of B-cell activation distinguish controllers from non-controllers

We investigated several immune parameters in order to define early immunological factors determining the disease course. First, we analysed lymphocyte subsets in blood by flow cytometry. In order to control for interindividual variation all data were related to individual pre-infection values. The percentage of CD4+ T-cells remained stable in all controllers. In contrast, the non-controllers showed a reduction in the percentage of CD4+ T-cells except for Mm9038 (Fig. 2a), which also maintained physiological levels of other immune parameters. The difference in CD4+ T-cell frequency between controllers and non-controllers became significant early after infection (8 and 12 weeks p.i.; \( P<0.05 \), Mann–Whitney test). Likewise, absolute CD4+ cell counts were reduced in the animals with detectable viral replication and dropped below 200 cells \( \mu \text{l}^{-1} \) in Mm1948 and Mm1961 (data not shown). Similarly, the proportion of CD29\(^{\text{high}}\)-expressing memory CD4+ T-cells, an early marker for disease progression (Blatt et al., 1995; Dolan et al., 1995; Kneitz et al., 1993), was decreased in monkeys with overt viral replication (Fig. 2b).

Interestingly, the strongest alterations after SIVmac239\(\text{DNU}\) infection were observed within the B-cell population. Whilst the proportion of B-cells in blood dropped in all animals at 2 weeks p.i. (Fig. 2c), a differential increase was observed during the following weeks. Controllers regained pre-infection levels 2 weeks later and maintained the levels, whereas in the non-controllers this population expanded continuously. The difference between the two groups reached significance at 8 weeks p.i. \( (P<0.05, \text{Mann–Whitney test}) \). Increased activation of B-cells was further investigated by a phenotypic analysis of these cells. Macaque B-cells can be

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**Fig. 2.** Lymphocyte subsets in SIVmac239\(\text{DNU}\)-infected rhesus macaques. Median values of controllers (blue) and non-controllers (red) are indicated by straight lines. Data of individual animals are shown by dots. Mm9038, which displays the phenotype of a viraemic controller, is shown by a dashed line. Asterisks mark significant differences between controllers and non-controllers (including Mm9038) calculated by the Mann–Whitney test \( (P<0.05) \). (a) Proportion of CD4+ T-cells normalized to mean of three values before infection. (b) Proportion of CD4+CD29+ T-cells normalized to mean of three values before infection. (c) Proportion of B-cells normalized to mean of three values before infection. (d) Proportion of CD80+CD21− B-cells normalized to mean of three values before infection.
subdivided into two mutually exclusive subsets according to the expression of CD21 and the co-stimulatory molecules CD80 or CD86 (Sopper et al., 1997). CD21high cells represent terminally differentiated cell (resting cells), whereas CD21low cells represent activated (differentiated) B-cells (Niino et al., 2009). In a subset of nine macaques, we determined levels of CD80+CD21−B-cells. Activated CD80+CD21− cells increased in non-controllers, whereas in the controllers these cells remained stable (Fig. 2d). The difference between controllers and non-controllers became significant by 16 weeks p.i. (P<0.04, Mann–Whitney U test). These findings indicate a strong activation of B-cells in the non-controllers.

SIV-binding antibody titres were also determined (Fig. 3). Early in infection (8 to 24/26 weeks p.i.), non-controllers had significantly higher Env (gp130)-binding antibody titres compared with controllers (P<0.05, Mann–Whitney test; Fig. 3a). In contrast, the kinetics of Gag (p27)-specific antibody titres were similar for both groups (Fig. 3b). Only late after infection did Gag antibodies titres start to decline in those animals with the highest viral load (Fig. 3a), consistent with the known prognostic value of low-Gag-binding antibodies (Putkonen et al., 1992; Weber et al., 1987).

No evidence for increased viral virulence after transfusion of blood from monkeys with progressing disease

Previous studies have shown that partial repair of defective nef genes is associated with increased viral load in some HIV-infected patients or SIV-infected macaques (Carl et al., 2000; Chakrabarti et al., 2003; Sawai et al., 2000; Whatmore et al., 1995). To investigate whether the nef deletion was still intact, nef PCR and direct DNA sequencing of the PCR products were carried out in the two macaques with the highest viral load, Mm1891 and Mm1948. We found no evidence of refilling of nef sequences, and the DNA sequences around the nef deletion site were identical between the infecting virus and those analysed in peripheral blood mononuclear cells (PBMCs) of the macaques progressing to disease (data not shown).

To investigate whether the virus had changed its virulence in monkeys with progressing disease, a transfusion experiment was performed. Aliquots of 5 ml of peripheral blood of Mm1891 or Mm1948 spiked with 10⁷ autologous lymph node cells were transfused into two naïve animals (Mm1891T and Mm1948T) at 54 weeks after donor infection. In order to avoid potential effects attributable to escape mutants that may have developed in the donor macaques, recipient animals carried MHC genotypes different from the donors and lacked MHC class I genes known to be associated with superior viral control such as Mamu-A1*01 or -B*17 (Table S1). However, one monkey (Mm1948T) carried MHC class II DRB alleles that have been reported to be over-represented in elite controllers (Giraldo-Vela et al., 2008).

After infection, viral RNA copy numbers declined steadily to undetectable levels in Mm1891T and at least by three orders of magnitude in Mm1948T during the observation period (Fig. 4a). The cell-associated viral load declined with time, and reached the detection level of the assay at 36 and 44 weeks p.i. (data not shown). Similar to the controllers, the percentage of CD4⁺CD29⁺ cells remained stable during the observation period (Fig. 4b). Likewise, the portion of B-cells did not change conspicuously, albeit an increase was later seen in infection in Mm1948T (Fig. 4c). Furthermore, in contrast to the donor monkeys, the transfused macaques developed high Gag and low Env antibody titres (Fig. 4d, e). In contrast to the donor macaques, which developed severe lymphadenopathy and other signs of AIDS-related symptoms, the two transfused animals displayed only mild symptoms at euthanasia (58 and 61 weeks p.i.) (Table 2). Considering the high degree of immune activation probably induced by the inoculation of foreign antigen, the transferred virus still retained the attenuated phenotype of the original clone.

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**Fig. 3.** SIV-specific serum antibody titres after infection with SIVmacΔNU: individual titres (reciprocal log10 dilution) against (a) SIVgp130 and (b) SIVp27 at the indicated time points. Controllers are shown in blue, non-controllers in red. gp130 titres differ between controllers and non-controllers significantly at 8, 16 and 24–26 weeks p.i., and are marked by asterisks (P<0.03, Mann–Whitney t-test). Titres from three macaques with the highest viral load are indicated by crosses.
MHC genotypes do not exclusively explain the differential disease progression

Genes encoded in the MHC class I region are important genetic determinants of disease progression in pathogenic SIV infection. Previously, we have identified MHC class I haplotypes and single MHC class I sequences associated with rapid, moderate and slow disease progression (Sauermann et al., 2008). MHC haplotypes were known for the nine macaques originating from the German Primate Center (DPZ) colony. The remaining animals from other breeding facilities were typed in detail by employing 49 MHC primer pairs specific for single MHC alleles or allelic lineages. Four of eight controllers, but none of the non-controllers, carried MHC class I alleles reported to be associated with slow or moderate disease progression (p < 0.02, x² test; Tables 1 and S1). Two controllers carried genotypes previously associated with rapid disease progression in pathogenic SIV infection. Furthermore, these two animals had MHC-identical siblings that had progressed rapidly to AIDS-like disease after infection with pathogenic SIVmac (Sauermann et al., 2008). In contrast, only one out of nine non-controllers carried MHC alleles associated with rapid disease progression. The remaining animals in both groups carried MHC class I sequences with hitherto undetermined association with disease progression after SIVmac infection, such as Mamu-B*43, -B*26, -B*27 or -A1*002 (Sauermann et al., 2008). The analysis of MHC class II DQB1 and DRB genes revealed five DQB1-DRB homozygous macaques that were able to control viral replication. In contrast, only three non-controllers were homozygous for their MHC DQB1-DRB genotype. Homozygous macaques express only one DQB1 gene and one or two different DRB genes, and thus present presumably a smaller repertoire of peptides as compared with heterozygous macaques. Interestingly, within the group of controllers, MHC class II homozygous macaques initially had a significantly higher viral load as compared with MHC class II heterozygous macaques (area under curve 0–20 weeks p.i.: P = 0.036, Mann–Whitney test, Fig. 5). The two Mamu-A1*001-positive macaques were equally distributed between the DQB1-DRB homozygotes and heterozygotes. This effect was not observed in macaques with median to high viral load.

TLR7 polymorphisms are not significantly associated with viral replication

A TLR7 variant has been found to be associated with a more severe clinical disease course in HIV-infected human patients (Oh et al., 2009). In SIV-infected rhesus macaques, we previously identified a TLR7 genotype (c.<17C, c.13G) associated with prolonged survival and lower set-point viral load compared with the other investigated TLR7 genotypes (Siddiqui et al., 2011). Six of eight macaques controlling viral replication carried the genotype c.<17C, c.13G associated with prolonged survival (75%) and four
DISCUSSION

The protective immune responses induced by infection with nef-deleted SIVs have been considered to be instructive for the development of an effective AIDS vaccine (Daniel et al., 1992; Koff et al., 2006; Whitney & Ruprecht, 2004). This appears to be of particular importance, as clear correlates of protection against HIV infection are only beginning to emerge (de Souza et al., 2012; Haynes et al., 2012). Interestingly, protection conferred by live attenuated viruses can be obtained in the presence of low or even absent adaptive immune responses (Mansfield et al., 2008; Stebbings et al., 2005; Wade-Evans et al., 2001). Recent reports have shown that antibody-dependent cell-mediated cytotoxicity and CD4⁺/CD8⁺ T-cell responses, especially in lymph nodes, are responsible for conferring protection against challenge with pathogenic SIV (Alpert et al., 2012; Fukazawa et al., 2012). An obstacle to using nef-deleted viruses for AIDS vaccine research in non-human primates is their varying replication pattern, especially in rhesus macaques of Indian origin, affecting vaccine efficacy, e.g. by fostering the generation of recombinant viruses (Gundlach et al., 2000; Reynolds et al., 2008), or by themselves causing AIDS-like disease in non-controllers (Hofmann-Lehmann et al., 2003).

In this study, our aim was to analyse whether immunological, host genetic factors or gross changes in the viral genome were associated with differential viral load and disease progression in rhesus macaques infected with SIVmac239ANU. Plasma viral RNA copy numbers were reduced initially in all macaques, but viral loads at set point finally showed considerable differences, with mostly non-detectable levels in controllers and plasma viral load >10⁴ copies ml⁻¹ in non-controllers. Controllers had stable CD4⁺ and B-cell counts, and did not display symptoms of immunodeficiency during the observation period. The non-controllers replicated SIVmac239ANU at moderate to high levels and the three with the highest plasma viral load had declining CD4⁺ T-cells. They were euthanized with signs of immunodeficiency, which can be related directly to the SIVmacΔnef infection. Plasma RNA copy numbers in controllers and non-controllers already differed significantly at peak viraemia. As at this point in time adaptive immune responses play only a minor role in controlling viral replication, the intrinsic capability to replicate the virus may have influenced the outcome of infection.

Non-controllers already had increasing numbers of activated B-cells in the early stages of infection, elevated SIV Env as well as lower SIV Gag antibody titres. Thus, B-cell responses deteriorated in the non-controllers. B-cell dysfunction represents a hallmark of HIV and SIV infection (De Milito, 2004; Moir & Fauci, 2009). Some effects of B-cell dysfunction, such as hyperactivation and impairment of immunoglobulin class switching, have been attributed directly to Nef (Moir & Fauci, 2010; Qiao et al., 2006; Swingler et al., 2008; Xu et al., 2009). As the macaques had been infected with a nef-deleted virus, B-cell hyperactivation seen in these monkeys could not have
been caused by Nef, but through a direct consequence of viral replication.

Our transfusion experiment has shown that the virus from immunocompromised monkeys retained an attenuated phenotype after passage in susceptible animals. Sequence analyses revealed that the virus had kept the nef deletion. Viral loads in transfused monkeys were reduced by at least 20-fold compared with those in the donor animals in the chronic phase of infection. No decrease of CD4⁺CD29⁺ T-cells was observed and the proportion of B-cells remained stable. Furthermore, both monkeys had rather low Env and Gag antibody titres similar to controllers. These findings contrast with other reports demonstrating that in vivo passage increases the pathogenicity of SIVmac or SHIV (simian-human immunodeficiency virus) (Holterman et al., 1999; Tan et al., 1999), or showing that the genome of viruses with impaired or deleted Nef function can acquire compensatory mutations that increase pathogenicity (Alexander et al., 2003). A difference from the other reports is, however, that the transfused macaques of this study received material from monkeys that had not developed AIDS-like symptoms. Although the transfused macaques did not show all features of perfect controllers, viral replication was clearly attenuated compared with the donor macaques. Therefore, we conclude that host factors played the major role in determining the extent of viral replication in this study.

We did not find a common strong genetic predictor of disease progression in Δnef-infected animals. Although MHC class I alleles associated with some control of SIVmac infection (Sauermann et al., 2008) were found exclusively among the controllers, two carried MHC class I genotypes associated with rapid disease progression in SIVmac-infected macaques. Similarly, genotyping of TLR7 variants described to be correlated with disease progression in pathogenic SIV infection (Siddiqui et al., 2011) did not provide clear associations in our study with nef-deleted virus.

Results only became significant when genetic and viral load data were related to protection from infection with SIVmac251. Four of five animals (Freissmuth et al., 2010) that did not strongly replicate the challenge virus SIVmac251 at necropsy were – in contrast to the other four controllers and the majority of non-controllers – homozygous for MHC class II and carried a TLR7 genotype associated with slow disease progression, but had divergent MHC class I genotypes. Thus, in accordance with other reports, cytotoxic T lymphocyte (CTL) responses may play only a minor role in protective immune responses after SIVmacΔnef infection (Mansfield et al., 2008; Stahl-Hennig et al., 2007; Stebbings et al., 2005). The fifth protected animal was a non-controller (Mm8637). The slight over-representation of the TLR7 polymorphism in protected macaques may be related to differential immune activation. The association of Mhc class II genotypes with protection may be linked to the fact that early viral replication in controllers was strongest in macaques homozygous at the MHC class II DQB-DRB region. The effect was probably masked in the non-controllers by the vigorous viral replication. This result matches in part observations that the extent of viral replication and/or the degree of attenuation determine protection against pathogenic SIVmac (Koff et al., 2006; Whitney & Ruprecht, 2004).

Among the non-controllers, only the macaque with the lowest peak viraemia was protected from infection with SIVmac251; others progressed to early signs of immune dysfunction or replicated finally SIVmac251. Moreover, in contrast to Fukazawa et al. (2012), we found no evidence that increased presence of SIV in lymph nodes before challenge was associated with protection. Our results suggest that a certain degree of early viral replication followed by an efficient immune response that most likely includes T-cell responses in lymph nodes was associated with protection. Further analyses will be required to investigate whether homozygosity for MHC class II genes or polymorphisms in genes linked to this region influence initial viral replication and protection. Moreover, viral replication may be determined by host factors which differ from those after pathogenic SIV infection. In the context of an infection with Nef-impaired or nef-deleted virus, the impact of some host factors may be altered because of the slower replication kinetics of the virus or because they are not inactivated by Nef, such as tetherin and cell-surface receptors, e.g. CCR5 or CXCR4 (Hrecika et al., 2005; Landi et al., 2011; Michel et al., 2006; Zhang et al., 2009). Identification of these host factors will provide further insights into the mechanisms associated with viral control.

**METHODS**

**Rhesus monkeys.** Nineteen rhesus macaques (M. mulatta) of Indian origin, seronegative for SIV, simian T-cell leukemia virus type 1 and D-type virus, were infected with SIVmac239ANU (Gundlach et al., 1997). Eleven animals were inoculated intravenously with 300 TCID₅₀ SIVmac239ANU. Six macaques were infected by atraumatic application of 10⁵ TCID₅₀ SIVmac239ANU onto the tonsils (Stahl-Hennig et al., 2007; Tenner-Racz et al., 2004). Another two monkeys were transfused with 5 ml citrated blood spiked with 10⁷ lymph node cells from two donor monkeys 54 weeks p.i. with SIVmac239ANU. The overall number of transferred cell-associated infectious units was 25 600 for 1891T and 21 440 for 1948T. The housing and treatment protocols of the DPZ follow the German Animal Welfare Act strictly, which in turn complies with European Union guidelines on the use of non-human primates for biomedical research. An external ethics board empowered by the Lower Saxony State Office for Consumer Protection and Food Safety approved all experiments under the project licences 509.42502/08-02.95 and 509.42502/08-13.98. Blood samples were drawn from monkeys which were anaesthetized intramuscularly with 10 mg ketamine (kg body weight)⁻¹. Twelve macaques were later inoculated with SIVmac251 as described previously (Freissmuth et al., 2010; Tenner-Racz et al., 2004).

**Cell-associated viral load and plasma viral RNA copy number determination.** The cell-associated virus load in peripheral blood and lymph nodes was determined by a limiting-dilution cocultivation assay as described previously (Stahl-Hennig et al., 1996; Stahl-Hennig et al., 1992). Viral RNA copies in plasma samples by
were determined by competitive quantitative RNA-PCR (Ten Haaft et al., 1998).

**Lymphocyte phenotyping and counting.** Three-colour flow cytometry was performed as described previously (Sopper et al., 2000). Briefly, 50 μl citrated blood was incubated with the antibodies at pre-titrated concentrations for 30 min at 4 °C. The following antibody combinations were used together with the anti-monkey CD3 antibody FN18 (biotin conjugated using standard techniques; M. Jonker, TNO, Rijswijk, The Netherlands) to determine T-cell subsets: CD29 (4B4, FITC conjugated; Coulter) and CD4 (OKT4, phycoerythrin (PE) conjugated; Ortho); CD8 (RPA-T8, FITC coupled; Pharmingen) and CD69 (L78, PE conjugated; Becton Dickinson); CD8–FITC and Ki-67 (PE conjugated, Dako). For phenotypic characterization of B-cells (CD20, 2H7; Cy-Chrome conjugated; Pharmingen) antibodies directed against CD21 (B-IV4, FITC conjugated; DPC) and CD80 (L307.4, PE coupled; Becton Dickinson) were used, which define different subsets (Sopper et al., 1997). After lysing of erythrocytes and fixation of the cells with FACs lysing solution (Becton Dickinson), bound biotinylated antibodies were detected with streptavidin-coupled Cy-Chrome (Pharmingen). For intracellular staining with Ki-67, cells were fixed using 3 % formaldehyde and membranes were permeabilized using 0.3 % Triton X-100. Cells were analysed on a FACScan flow cytometer (Becton Dickinson) using Lysis II and CellQuest software. Quadrants were set according to the staining pattern obtained with isotype-matched control antibodies, except for the bimodal distribution of CD29 where markers were set between the two CD29low- and CD29high-expressing populations. For the determination of absolute numbers of lymphocytes, the percentage of the respective population within a forward and side light scatter gate including CD3+ T-cells, CD20+ B-cells and CD3+CD8+ NK cells (total lymphocytes) was calculated. This proportion was combined with the absolute number of lymphocytes obtained using a Coulter counter.

**SIV-binding antibodies.** To measure humoral SIV-specific responses, a standard ELISA for the detection of antibodies against the SIV polypeptides gp130 SU and p27 capsid antigen (Stolte-Leeb et al., 2006) in a limiting-dilution format was performed. For antigen coating, recombinant monomeric SIVgp130 (EVA670) and SIVp27 (EVA643) kindly provided by the Centre for AIDS Reagents, National Institute for Biological Standards and Control (NIBSC), UK, were used. ELISA titres refer to the serum dilution yielding a twofold higher absorbance compared with that obtained with sera collected before infection.

**Analyses of viral nef sequences.** To investigate whether the nef deletions were still present in the animals with a high viral load (Mm1948 and Mm1891), DNA was extracted from lymphocytes obtained at 64 and 88 weeks p.i. nef PCR was performed as described previously (Gundlach et al., 1997). Size analysis was performed by agarose gel electrophoresis and DNA sequence analysis of the purified PCR products was performed by SeqLab.

**Genotyping.** Typing of the DQB1 alleles was performed by PCR-RFLP or by DNA sequence analysis of exon 2 as described previously (Vigon & Sauermann, 2002). For DRB typing, PCR products of exon 2 were separated by denaturing gradient gel electrophoresis, followed by DNA sequence determination of the eluted and reamplified PCR products (Khazand et al., 1999). MHC class I typing with 49 MHC class I typing primers was performed essentially as described previously (Sauermann et al., 2008).

Typing for TLR7 single nucleotide polymorphisms c.−17C>T and c.13G>A was performed as described previously (Siddiqui et al., 2011).

**Statistical analysis.** GraphPad Prism version 5 was used for statistical analysis. The Mann–Whitney U test was applied for comparisons between groups. Correlations were calculated using Spearman rank correlation. The statistical tests employed are indicated along with each P value.

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**REFERENCES**


