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The Effect of *nef*-Deleted SIV Administration on Disease Progression in SIV-Infected Rhesus Macaques

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Abstract

The effect of a live *nef*-deleted $SIVmac_{239}$ construct (SIVΔ*nef*) was evaluated in rhesus macaques infected with wild-type $SIVmac_{239}$. Rhesus macaques were inoculated by intravenous administration of 100 tissue culture infectious doses 50% ($TCID_{50}$) of $SIVmac_{239}$. All animals had detectable viremia at 1 week post-inoculation (pi), with peak viremia (6 to 100 million copies viral RNA/ml plasma) two to three weeks pi. From week 4 to week 8 pi, virus levels ranged from ~ 10^4 to ~ 10^6 copies/ml, which is the expected range for $SIVmac_{239}$. MHC type 1 Phenotyping and week 4 viral load data were used to stratify the animals into 3 groups, which all received 2 consecutive intravenous injections of Placebo or SIVΔnef at weeks 8 and 10 pi. The control group received Placebo vehicle (RPMI1640 medium). The Low Dose group received 4e5 $TCID_{50}$ units/dose of SIVΔ*nef*, while the log-fold High Dose group received 4e6 $TCID_{50}$ units/dose. Animals were monitored daily for clinical signs. At defined regular time points, weight and body temperatures were recorded and blood and urine samples were collected for viral load analysis, clinical haematology, clinical chemistry, urinalysis and immunological assay. No obvious differences were observed in any parameters between the three experimental groups. We conclude that SIVΔ*nef* injection in rhesus macaques infected with wild-type SIV did not increase the progression to disease compared to Placebo control.

We also investigated whether intradermal injection of rhesus macaques with nef-deleted SIV is a suitable route to induce infection with this live-attenuated SIV. Two animals that received a high-dose intradermal injection of SIVΔ*nef* became infected and had a pattern of viremia that is indistinguishable from historical data for animals inoculated intravenously with this virus. These findings confirm the attenuated phenotype of SIVΔ*nef*.

Keywords:
SIV *nef* deletion; Live attenuated virus; Viral kinetics; Intradermal inoculation

Introduction

A live attenuated form of $SIVmac_{239}$ containing a deletion in the *nef* gene has been used to study the role of *nef* in lentivirus viral infectivity and disease progression [1, 2]. The *nef* gene is expressed early in SIV infection to promote the replication of the viral genome [3-7] and in maintaining viral load *in vivo* [8].

The observed enhancement of viral infectivity was independent of cell line used, multiplicity of infection and number of virus replication cycles [9]. Superior growth and infectivity was observed in both human and macaque primary cell cultures infected with SIV and with recombinant HIV expressing SIV *nef* [10, 11].

The mechanism by which SIV *Nef* enhances viral infectivity is not completely understood, however, *Nef* has been shown to down regulate CD4 and MHC1-1 expression, as well as interfere with CD8 T-cell response in monkeys [12].

Additional functions attributed to *Nef* include interfering with the endocytosis machinery, directing the activation status of infected cells and disrupting the actin cytoskeleton to render cells more permissive to viral infection [13].

Based on the many roles of *Nef*, together with the findings that *nef*-deficient mutants produce much lower viral loads accompanied by decreased pathogenicity, demonstrates that *nef* is a key factor in viral fitness and suggest that *nef*-deficient SIV can act as a live attenuated virus vaccine [8].

Traditionally, to elucidate the characteristics of a live attenuated SIV virus, it is inoculated intravenously into SIV naïve monkeys [14-16]. Most animals develop detectable viremia a few weeks post-inoculation [14, 16] and an immune

response to the attenuated virus is often seen during this viremia [14, 17].

Although infection of rhesus macaques by intravenous route are used to study viral pathogenesis and disease progression, additional routes of virus inoculation should be established in this model to fully understand the features of a live attenuated virus.

This paper describes the effect of intravenous inoculations of SIVΔ*nef* virus into monkeys infected with $SIVmac_{239}$. We will also discuss the ability of SIVΔ*nef* to infect rhesus macaques through an intradermal route.

Materials and Methods

Animals

Rhesus macaques from the SIV-naïve colony at California National Primate Research Centre (CNPRC) were selected based on age range of 4 to 7 years and body weight range of 6-10 kg, with size as uniform as possible within genders. The animals were pair-caged in a BSL 2+ facility during the study period.

Body weight and temperature was recorded regularly. Blood samples were collected for clinical haematology, clinical chemistry, viral and immunological assays. Urine samples were collected by cystocentesis for urinalysis.

All animals were housed at the California National Primate Research Centre (CNPRC) in accordance with American Association for Accreditation of Laboratory Animal Care Standards and the "Guide for the Care and Use of Laboratory Animals," by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Resource Council [18].

The study was approved by the Institutional Animal Care and Use Committee of the University of California Davis.

Determination of host genetics

MHC typing for 2nd class I alleles (Mamu-A*01, A*08, B*01, B*08, B*17, B*29) was performed by the Veterinary Genetic Laboratory (University of California, Davis) and the AIDS Vaccine Research Laboratory (University of Miami) using methods previously described [19-21]. Animals were assigned to have random distribution of MHC alleles and comparable virus levels at week 4 pi.

Infection with $SIVmac_{239}$

$SIVmac_{239}$ stock (batch number 2011) was propagated on rhesus PBMC and had a titer of 50,000 $TCID_{50}$ per ml. All animals were injected with $SIVmac_{239}$ by intravenous route with 100 $TCID_{50}$ at time 0.

Virus preparation and inoculation of SIVΔ*nef* by intravenous route

nef-deleted $SIVmac_{239}$ plasmid was obtained from NIH AIDS Reagent Program (catalog #12246), and virus stocks were produced in CEMX174 cells. The *nef* deletion was confirmed by sequencing and viral stock with titre of 3.2×10^6 $TCID_{50}$ per ml was selected for macaque infection studies. At 8 and 10 weeks pi, animals were injected with either High Dose SIVΔnef (4×10^6 $TCID_{50}$), Low Dose SIVΔ*nef* (4×10^5 $TCID_{50}$), or Placebo consisting of RPMI 1640 medium.

Inoculation of SIVΔ*nef* by intradermal route

Two animals were inoculated intradermally with 0.8 ml of 3.6 million $TCID_{50}$ per ml. Due to the limited volume that can be administered intradermally, animals were inoculated at 8 injection sites of 0.1 ml each; thus, the total dose administered was approximately 3 million $TCID_{50}$.

Clinical monitoring and sample collection

Routine daily observations were performed according to CNPRC SOP # F01: “Morning Health Check of Indoor Animals”. A subjective, non-quantitative observation of food consumption was made as part of the animal husbandry monitoring. Physical exams, including measurement of body weight and temperatures were performed at every study period.

At specified study dates, the sedation and physical exams were combined with the collection of samples (blood and urine) for viral load determination, haematology, clinical chemistry, flow cytometry, cryopreservation of serum, and urinalysis.

Clinical laboratory analysis

Serum was tested for a standard clinical chemistry panel. Components measured include sodium, potassium, chloride, total carbon dioxide (TCO_2), inorganic phosphorous, calcium, BUN (blood-urea-nitrogen), creatinine, glucose, total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK), alkaline phosphatase, gamma glutamyl transferase (GGT), lactate dehydrogenase, triglycerides, cholesterol, total and direct bilirubin.

A sample of whole blood were collected for red blood cell (erythrocyte) count, white blood cell (differential) count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet counts, plasma protein and fibrinogen.

Urine was collected via cystocentesis according to standard operating procedures. It was analysed by Clinical Laboratory staff at CNPRC for specific gravity by a refractometer; the pH and the presence of protein, glucose, ketone, bilirubin, and occult blood was determined using Multistix 10 SG strips (Siemens Healthcare Diagnostics). Urine sediments were examined microscopically.

Plasma viral load

SIV RNA levels in the plasma samples were tested by RT-PCR assay for SIV gag, as previously described [22]. The remaining blood was subjected to density gradient (Lymphocyte Separation Medium) centrifugation to isolate peripheral blood mononuclear cells (PBMC). Statistical analyses were performed using 1-way ANOVA for viral loads at week 4. A value of P of <0.05 was considered statistically significant.

Phenotyping of lymphocyte populations

An aliquot of EDTA-anti-coagulated blood was combined in a single tube with the following antibodies, all from BD Biosciences (San Jose, CA): Anti-CD3-Pac-blue (clone SP34-2), anti-CD4-PerCP-Cy5.5 (clone L200), anti-CD8-VK500 (clone SK1), anti-CD14-FITC (clone M5E2), anti-CD16-PE-Cy7 (clone 3G8), anti-CD20-APC (clone L27) and anti-CD28-PE (clone L293). Multi-parameter flow cytometric analysis was used to test for the expression of CD3, CD4, CD8, CD14, CD16 and CD20 to determine the percentage of total T cells (CD3+), helper T cells (CD4+), cytotoxic T cells (CD8+), monocytes (CD14+), NK cells (CD16+) and B cells (CD20+).

Criteria for euthanasia and necropsy

Euthanasia of animals with simian AIDS was determined by established criteria of one or more of the following clinical observations indicative of a severe life-threatening situation: weight loss of >15% in 2 weeks or >25% over any time course; chronic diarrhoea or other opportunistic infections unresponsive to treatment; inability to maintain body heat or fluids without supplementation; obtundation; neurologic deficits; persistent, marked hematologic abnormalities, including anaemia (<20%), thrombocytopenia with petiachiae or ecchymosis, and hypoproteinemia with edema.

Results and Discussion

Inoculation of SIVΔ*nef* virus in SIV infected rhesus macaque

Eighteen rhesus macaques infected with $SIVmac_{239}$ at day 0 were assigned to three study groups, Group 1 Placebo, Group 2 Low Dose SIVΔ*nef*, and Group 3 High Dose SIVΔnef **(Table 1)** based random distribution of MHC alleles and comparable viral loads at week 4 pi. Twelve out of eighteen animals tested positive for MHC alleles A08, B01, B08, B17 and B29 is shown in **Table 2**. None of the animals were positive for the protective allele A01.

To randomly distribute the MHC alleles, a value of +1 was given to protective alleles (A01, B08, B17), -1 to negative alleles (B01) and 0 to neutral alleles (A08, B29). The total MHC score for female rhesus macaque in the Placebo, Low, and High Dose group were 0, 0 and -1, respectively. The total MHC score for male animals in those groups were 0, 0, and +1. Viral loads in SIV infected rhesus macaques were also compared at 4 weeks pi. Mean viral load (Log 10) in female animals were 5.26, 5.26 and 5.32 (p=0.97) for Placebo, Low and High Dose groups. For male rhesus macaques, mean viral loads in those groups were 5.41, 5.33 and 5.18 (p=0.88). The viral loads in all animals were comparable regardless of MHC alleles at 4 weeks pi. This suggests that these genotypes did not influence the acute-phase plasma virus concentrations.

Table 1 Overall experimental design.

Groups	M	F	SIVmac239 Week 0 IV route	Study Agent Week 8 and 10 IV route	Total Study Phase Duration
Placebo	3	3	100 $TCID_{50}$ units	Placebo	16 weeks
Low Dose	3	3	100 $TCID_{50}$ units	Δ*nef* $SIVmac_{239}$, 4e5 TCID50	16 weeks
High Dose	3	3	100 $TCID_{50}$ units	Δ*nef* $SIVmac_{239}$, 4e6 TCID50	16 weeks

Table 2 MHC typing and distribution of animals into Placebo, low and high dose group.

Group	Animal ID	Sex	MHC typing							Log of viral RNA/ml plasma (week 4)
			A01	A08	B01	B08	B17	B29	MHC Score*	
Placebo	38890	F	-	-	+	-	+	+	0	4.93
Placebo	38902	F	-	-	-	-	-	-	0	5.3
Placebo	38924	F	-	-	-	-	-	-	0	5.56
Low	39139	F	-	-	-	-	-	-	0	5.36

Low	39204	F	-	-	+	-	+	+	0	4.94
Low	39600	F	-	+	-	-	-	-	0	5.48
High	39756	F	-	-	+	-	-	-	-1	4.79
High	39944	F	-	+	-	-	+	+	1	5.59
High	40184	F	-	+	+	-	-	-	-1	5.58
Placebo	38092	M	-	-	+	-	-	-	-1	5.4
Placebo	39330	M	-	-	-	-	+	-	1	5.56
Placebo	40233	M	-	-	-	-	-	-	0	5.28
Low	39473	M	-	+	-	-	-	-	0	5.45
Low	39616	M	-	-	-	-	+	+	1	5.11
Low	40358	M	-	-	+	-	-	-	-1	5.43
High	39863	M	-	-	-	-	-	-	0	5.36
High	40062	M	-	-	-	-	-	-	0	6.04
High	40124	M	-	+	-	+	-	-	1	4.15

At 8 and 10 weeks pi, animals were inoculated intravenously with either RPMI media, 4 × 10^5 $TCID_{50}$ or one-log fold higher dose 4 × 10^6 $TCID_{50}$ of the attenuated virus. Animals were monitored for changes including clinical haematology, clinical chemistry, plasma viral loads and lymphocyte cell counts.

In all animals, weights and temperatures were collected regularly throughout the study duration. No significant differences in temperature were observed and the weight of most animals remained close to pre-infection values or increased (data not shown).

In other clinical observations, animal 40358 from the Low Dose group had two reports of mild dermatitis prior to $SIVmac_{239}$ infection 4 months apart. After inoculation with $SIVmac_{239}$, moderate dermatitis was observed at 4 weeks, and topical treatments were applied starting at 12 weeks pi. Animal 38890 from the Placebo group had intermittent reports of mild dermatitis throughout the study, but no special treatments were given.

Plasma viral loads in SIV-infected rhesus macaques did not change after two intravenous inoculations with low or high dose of SIVΔ*nef* compared to Placebo control **(Figure 1a)**. One animal in the Placebo group, 38890 had progressively low viral loads over time with a 3 log reduction in viral load by 16 weeks pi.

This animal was positive for Mamu-B17 and Mamu-B29 allele, which has been demonstrated to show a degree of viral control after infection with pathogenic $SIVmac_{239}$ [23]. Animals 39204, 39944 and 39616 was also positive this allele but did not demonstrate a reduction in viral loads.

This is consistent with previous report, in which only 26% of B17 and B29 positive rhesus macaques infected with $SIVmac_{239}$ showed elite control of virus [23]. No differences were seen in plasma viremia between Placebo or SIVΔ*nef* inoculated animals **(Figure 1b)**, which is consistent with historical data of $SIVmac_{239}$ infection [24-26].

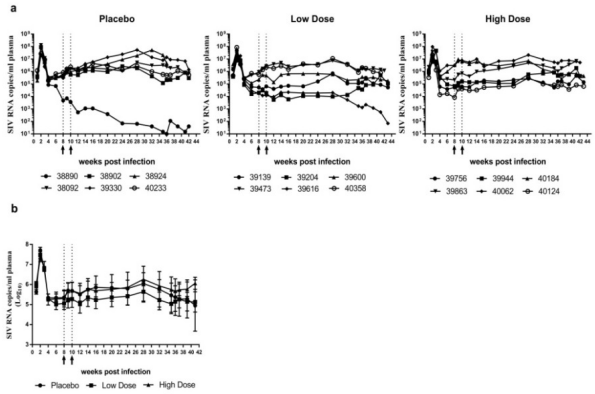

Figure 1 Viral kinetics in rhesus macaque infected with SIVmac239 at day 0 (a) Plasma viral loads in animals that received Placebo, low dose or high Dose. At 8 and 10 weeks pi (arrows), low and high dose groups were inoculated with SIVΔ*nef*. (b) Mean viral load of groups. Error bars represent standard error of measurement. Plasma viral loads measured as RNA copies/ml of plasma was determined by real-time RT-PCR assay for SIV gag RNA).

Acute infection with $SIVmac_{239}$ was characterized by a marked decline in B cells in the peripheral blood in Placebo, Low, and High Dose groups with 47.6±21.2%, 60.1%±28.5% and 49.5%±11.4% decline, respectively, at 2 weeks pi compared to baseline levels. However, there was a rapid recovery of peripheral B cells with restoration to pre-infection levels by 4 to 6 weeks pi **(Figure 2)**.

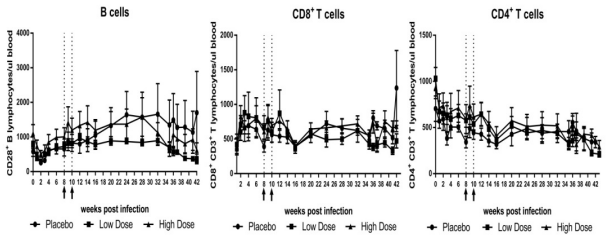

Figure 2 Absolute B cells, CD8 T cells, and CD4 T lymphocyte counts in peripheral blood. At 8 and 10 weeks pi (arrows), animals received either Placebo or Δnef SIVmac239 (Low Dose or High Dose). Error bars represent standard error of measurement.

Total T lymphocyte counts remained steady during the course of the infection, including steady state CD8 T lymphocytes. In addition, there were no changes in NK and monocyte levels. A decline in peripheral blood CD4 T lymphocyte was observed in during the first 8 weeks pi, followed by a gradual decline up to 43 weeks pi in all groups. CD4 T lymphocyte decline is an important marker of disease progression, and is consistently observed in $SIVmac_{239}$ rhesus macaque models for AIDS [27, 28].

Several parameters indicative of kidney function (BUN, creatine) and liver function (ALT, AST, GGT) and immune system (WBC), are shown in **(Figure 3a)**.

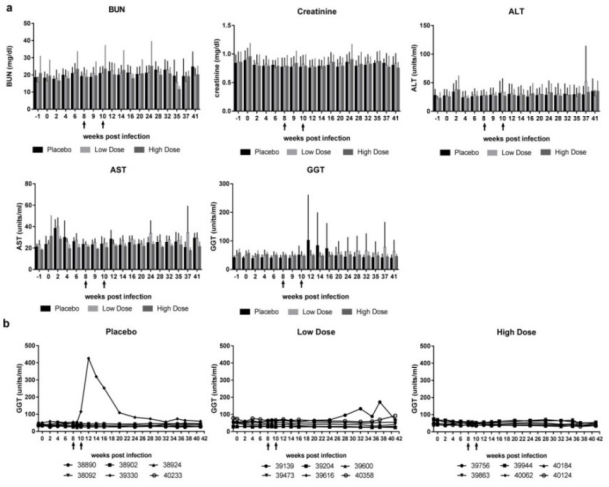

Figure 3 Clinical chemistry in infected rhesus macaques. (a) Parameters of kidney function (BUN, creatinine) and liver function (ALT, AST, GGT) from Placebo, Low Dose, and High Dose groups. Error bars represent standard deviation from mean. (b) GGT levels in individual animals. Arrow bars indicate inoculation of either RPMI media (Placebo) or SIV Δ*nef* (Low and High Dose).

In most animals, the ranges were within the normal values for clinical haematology, chemistry, and urinalysis tests as described in the methods section. In one animal from the Placebo group, 39300 developed increased levels of GGT, alkaline phosphatase and C-reactive protein from week 10 onwards.

Animal 39139 inoculated with low dose SIVΔ*nef* also had increased levels of GGT starting a 20 weeks pi **(Figure 3b)**. This is indicative of hepatobiliary disease, which is a common complication of SIV infection.

Study animals were continually monitored during chronic phase of infection until the progression of simian AIDS in some animals. Placebo animal 38924 showed signs of terminal SIV infection and was necropsied by 36 weeks pi. The animal had a clinical history of diarrhoea and gradual weight loss of 25% compared to pre-infection weight.

Two other animals met the criteria for euthanization at week 41. Animal 40358 from the Low Dose group had reduced appetite and developed respiratory distress and animal 39863 from the High Dose group showed severe anaemia and icterus. The study was concluded by 43 weeks post infection, and all animals had signs of advanced SIV infection including lymphadenopathy and spleenopathy.

The inoculation of SIVΔ*nef* during the early chronic phase of infection in the Low and High Dose groups did not influence the progression of disease in these animals compared to Placebo. Inoculation of SIVΔ*nef* did not change viral loads in SIV wild type-infected rhesus macaques compared to Placebo group.

CD4 counts also steadily declined and clinical symptoms were absent or mild. The lack of changes suggest that inoculation of SIVΔnef at 8 and 10 weeks post infection did not modify or accelerate the outcome of wild type infection.

Infection with SIVΔ*nef* virus by intradermal route

Two animals (39423 and 40307) were inoculated intradermally with SIVΔnef. Both animals were viremic starting one week after inoculation **(Figure 4a)**, with peak levels at 1 or 2 weeks (130,000 to 440,000 SIV RNA copies/ml). By 10 weeks pi, plasma viremia declined to below the limit of detection (<30 copies/ml; animal 39423) to 3,700 copies/ml (animal 40307).

These plasma levels are indistinguishable from those described previously in the literature for animals infected intravenously with SIVΔ*nef*, namely peak levels of ~ 10^4 to 10^5 SIV RNA copies/ml and viral set point at week 10 ranging from undetectable to ~ 10^3 SIV RNA copies/ml plasma [16, 29]. In accordance with these moderate virus levels, animals did not exhibit any clinical signs during the observation period. In comparison to wild type $SIVmac_{239}$, the two animals infected with the attenuated strain had a two log decrease in plasma viral load **(Figure 4b)**. This finding confirms the infectivity of SIVΔnef by an intradermal route.

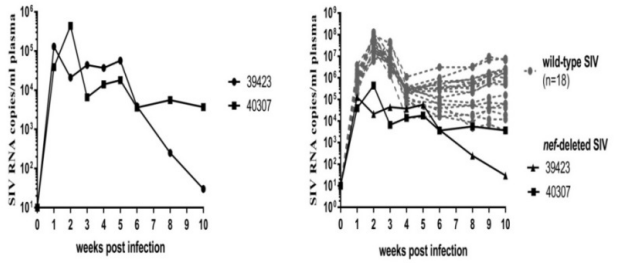

Figure 4 Plasma viremia of 2 animals inoculated intradermally with SIVΔ*nef* (a) SIV RNA copies/ml in animals 39423 and 40307 for up to 10 weeks pi. The limit of detection for this assay is 30 copies/ml. (b) Comparison of plasma viremia of SIVΔ*nef* with wild type $SIVmac_{239}$.

In conclusion, the study demonstrates the attenuated phenotype of SIVΔ*nef* virus and further understanding of SIVΔ*nef* virus may lead to vaccine constructs that can perhaps alter wild type SIV viral infectivity and disease progression.

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