厚生労働科学研究費補助金（エイズ対策研究事業）
分担研究報告書

霊長類エイズモデルの粘膜部位における感染動態と免疫応答

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研究要旨　相同組換えを利用して親株の持つ遺伝的多様性を再構築できる簡便で有用な新規組換えウイルス作製技術を確立した。これによりアカゲザルで良く増殖するCCR5指向性クレードC型臨床分離HIV-1株Envを持つ新規SHIV-97ZA012を短期間で作製することができた。今後本手法を用いて、サルで高い複製能を保持する種々の新規モデルウイルスを得ることによって、AIDS病態の解明とワクチンや薬剤の開発に貢献するものと期待される。

A. 研究目的

本研究の目的は、様々な病態を呈するサルエイズ発症モデルの粘膜部位におけるウイルス感染動態と免疫細胞応答について統合的に解析することにより、エイズウイルスの主要な標的臓器として注目されながらヒトでは解析が困難な粘膜部位における病態形成機構を解明すること、そしてサルエイズモデルによる新規予防・治療法開発の為の粘膜感染病態に基づく評価基準を確立することである。米国をはじめとする先進諸国において種々の抗エイズ薬の開発が積極的に行われ、それらを組合せた多剤併用療法(HAART)の導入によりエイズ感染者の血液中のウイルス量を減少させることが可能となった。しかし、HAART によりエイズ発症遅延効果は得られたが、エイズを根本的に治療する方法は未だ確立されていない。これまでは、基本的に感染者の末梢血中のウイルス量を減少させる効果を基準に治療法開発が行われてきた。しかし、エイズ根本治療法を開発する為には、エイズウイルスが体内のどこでいつどのように増殖しているのか、深部臓器でどんな免疫応答が起こっているのか、エイズウイルスの最も重要な標的部位がどこなのか等を明らかにする必要がある。最近、HIV-1 に類似のサルウイルス(SIV)を用いた研究によりエイズの標的臓器として腸管が重要であることが示され、エイズ患者においても腸管の重要性が示唆されている。一方、我々は外皮蛋白遺伝子を中心とした約半分のゲノム領域を HIV-1 のものと置き換えた SIV/HIV-1 キメラウイルス(SHIV)の作製によりサルエイズ感染・発症モデル系を確立し、これまでに様々な病態を呈する SHIV 感染性分子クローンを得ている。本研究により、サルエイズモデルの前臨床試験としての有用性が高まり、新しい評価基準に基づくエイズ根本治療法の開発が促進されるものと期待される。

B. 研究方法

アカゲザルを用いたエイズ発症モデル系の粘膜部位におけるウイルス感染動態と免疫細胞応答について、統合的な解析を行うことにより粘膜感染病態形成機構を解明し、サルエイズモデルによる新規治療法開発の為の粘膜感染病態に基づく評価基準を確立する。具体的には、1）感染サルの粘膜部位におけるウイルス増殖部位や潜伏部位等の感染動態について詳細に解析する。2）感染サルの粘膜や深部リンパ系組織における免疫細胞応答について詳細に解析する。3）感染サルの腸管をはじめとする全身の深部組織における病変の病理組織学的解析を行う。以上の解析を統合的に行うことにより、エイズウイルス感染サル個体の粘膜感染病態形成における最も重要な標的細胞群を特定し、ウイルス制御に有効に働く粘膜免疫機構を明らかにする。

（倫理面への配慮）

動物実験に当たっては、「研究機関等における動物実験等の実施に関する基本指針」に基づいた「京都大学における動物実験の実施に関する規定」を遵守する。当施設におけるアカゲザルの飼養については、「特定外来生物による生態系等に係わる被害の防止に関する法律」の規定に基づき、環境大臣より許可

を受けている。また、「感染症の予防及び感染症の患者に対する医療に関する法律」の輸入禁止地域等を定める省令に基づき輸入サル飼育施設の指定を受けている。「動物の愛護及び管理に関する法律」も遵守する。また、組換えSHIV感染実験については第二種使用等をする間に執る拡散防止措置について大臣確認されている。

C．研究結果

HIV-1はヒトとチンパンジーにしか感染しないことから、サル免疫不全ウイルス(SIV)とHIV-1のゲノムの一部を組換えたサルヒト免疫不全ウイルス(SHIV)がエイズのモデル系として使用されてきた。これまでに作製されたSHIVは分子クローン由来であるが、HIV-1は元来、多様性を保持した変異集団であり、このことがウイルスの適応度を高める要因の一つと考えられる。また、HIV-1の感染にはCD4の他にケモカイン受容体が必要であり、CCR5を利用するR5型ウイルスが、感染伝播と感染個体内での病態に重要なウイルスと考えられるが、既存のSHIVはCXCR4を使用するX4型ウイルスが多かった。一方、全ゲノムの93%がHIV-1で構成されサルに感染しうるHIV-1-NL-DT5Rが足立らによって構築されたが、このウイルスはX4型であり、サルにおける増殖能はまだ不十分である。そこで本研究では、サル個体内で馴化させることにより増殖能が向上し、遺伝的多様性を蓄積したR5型SHIV-MK38のenv領域をNL-DT5Rに組み込んだ新規ウイルスDT5R-MK38を相同組換え法により作製した。遺伝子解析を行ったところ、DT5R-MK38の組換えポイントは重複領域内に複数箇所存在し、SHIV-MK38の多様性の一部を保持していた。独立に作製したウイルス間でMK38の多様性の異なる系統を継承することがわかり、それらを混合することにより、元のMK38の遺伝的多様性を再構築できるものと考えられた。このウイルスをアカゲザルの末梢血単核球(PBMC)を用いて順化を試みたところ、CD8を除去したアカゲザルPBMCで安定して増殖するようになった。

前述したように、これまで主に作製されてきたSHIVは、CXCR4を使用するX4型であり、CCR5指向性である大多数のHIV-1とは病態が異なる事が分かっている。また、世界中のHIV感染者の約60%がcladeCウイルスに感染しているが、現存するcladeCのSHIVは数少ない。そこで本研究では感染伝播と病原性に深く関与しているCCR5指向性clade C HIV-1株のenv領域を持つSHIVの作製を行った。前述の細胞の相同組換え機構を利用した新規組換え技術を用いて、clade B SHIV-KS661をバックボーンとしてclade C HIV-1臨床分離株のenv領域を持つSHIVを作製した。シークエンス解析で組換え部位の特定を行い、本研究で採用した組換え技術により、clade C HIV-1 envを持つ新規SHIVの生成を確認した。作製した新規SHIV及び病原性分子クローン化ウイルスSIVmac239をアカゲザル末梢血単核球(PBMC)に接種後、培養上清中の逆転写酵素活性を経時的に評価したところ、新規SHIVはSIVmac239と同程度に複製した。低分子阻害剤を用いた共受容体指向性試験により、新規SHIVは、CCR5指向性であることを確認した。アカゲザル肺胞マクロファージでの複製能を評価したところ、新規SHIVはマクロファージ指向性である事を明らかにした。３頭のアカゲザルにこのSHIVを接種し、血漿中ウイルスRNA量、末梢血中CD4陽性T細胞数、肺胞中CD4/CD3陽性T細胞の割合の変動から、個体レベルのウイルス複製及び病原性を評価した。（１）新規SHIVはサル個体内において高力価で複製した。（２）感染サルの末梢血CD4陽性T細胞を減少させなかったが、CCR5指向性ウイルスが標的とするエフェクターメモリーCD4T細胞が多数分布するエフェクターサイトの一つである肺胞において、顕著にCD4陽性T細胞を減少させた。

D．考察

従来の制限酵素等を用いてプラスミド上で行うSHIV作製法は、組換えポイントが制限酵素認識部位に制約され、必ずしも最適な組換え部位とは限らず、感染性ウイルス自体を得ることが困難で、新規ウイルスを産生させるまでに数ヶ月ー数年程度の時間を要した。今回確立した相同組換え現象を利用した新規SHIV作製技術は、操作が簡便で、ウイルスに適した組換えポイントが選択される可能性を高め、親株の多様性を保持した状態で１週間程度の短期間で新規ウイルスを得ることができる。この新規組換えウイルス作製技術により、従来より短期間で、サル個体における複製能の高い新規SHIVの作製に成功した。今後この技術を用いて、有用な新規サルエイズモデルを確立できるものと期待される。

E．結論

相同組換えを利用して親株の持つ遺伝的多様性を再構築できる簡便で有用な新規組換えウイルス作製技術を確立した。これによりアカゲザルで良く増殖するCCR5指向性クレードC型臨床分離HIV-1株Envを持つ新規 SHIV-97ZA012 を短期間で作製することができた。今後本手法を用いて、サルで高い複製能を保持する種々の新規モデルウイルスを得ることによって、AIDS病態の解明とワクチンや薬剤の開発に貢献するものと期待される。

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G．知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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IV. 研究成果の刊行物・別刷（抜粋）

Protection of Macaques with Diverse MHC Genotypes against a Heterologous SIV by Vaccination with a Deglycosylated Live-Attenuated SIV

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Abstract

HIV vaccine development has been hampered by issues such as undefined correlates of protection and extensive diversity of HIV. We addressed these issues using a previously established SIV-macaque model in which SIV mutants with deletions of multiple gp120 *N*-glycans function as potent live attenuated vaccines to induce near-sterile immunity against the parental pathogenic SIVmac239. In this study, we investigated the protective efficacy of these mutants against a highly pathogenic heterologous SIVsmE543-3 delivered intravenously to rhesus macaques with diverse MHC genotypes. All 11 vaccinated macaques contained the acute-phase infection with blood viral loads below the level of detection between 4 and 10 weeks postchallenge (pc), following a transient but marginal peak of viral replication at 2 weeks in only half of the challenged animals. In the chronic phase, seven vaccinees contained viral replication for over 80 weeks pc, while four did not. Neutralizing antibodies against challenge virus were not detected. Although overall levels of SIV specific T cell responses did not correlate with containment of acute and chronic viral replication, a critical role of cellular responses in the containment of viral replication was suggested. Emergence of viruses with altered fitness due to recombination between the vaccine and challenge viruses and increased gp120 glycosylation was linked to the failure to control SIV. These results demonstrate the induction of effective protective immune responses in a significant number of animals against heterologous virus by infection with deglycosylated attenuated SIV mutants in macaques with highly diverse MHC background. These findings suggest that broad HIV cross clade protection is possible, even in hosts with diverse genetic backgrounds. In summary, results of this study indicate that deglycosylated live-attenuated vaccines may provide a platform for the elucidation of correlates of protection needed for a successful HIV vaccine against diverse isolates.

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Introduction

Molecular epidemiological studies have revealed the existence of an extensive degree of diversity of HIV-1 isolates [1]. HIV-1 is classified in three major groups (M, N, O) based on their geographical origin. Group M represents the predominant HIV-1 circulating through the world and has been divided into more than 10 subtypes (clades) as well as increasing number of circulating recombinant forms (CRF) primarily due to error-prone viral reverse transcriptase and the occurrence of super-infections. This diversity is continuously expanding worldwide and is a major obstacle for the successful development of an AIDS vaccine. While the generation of a vaccine capable to prevent transmission of HIV isolates endemic in a particular area remains an unfulfilled task, protection against phylogenetically distant viruses represents an even more formidable hurdle. The failure and dismal success of HIV-1 vaccine trials that have been conducted so far has prompted a re-emphasis for more basic studies concerning vaccine

design against heterologous challenge viruses, which can at present only be addressed in a macaque model. One of the pre-conditions for the objective assessment of the protective efficacy against a heterologous strain would be that the macaque model used should have the capacity to confer sterile or near-sterile immunity against the homologous virus challenge.

SIVmac239 infected rhesus macaques gradually develop AIDS after a variable period of chronic infection. In order to investigate the role and function of the glycan shield of the viral envelope, we previously developed a panel of deglycosylated mutants from this pathogenic SIVmac239 backbone [2]. Among these mutants, one mutant with five *N*-glycans deleted (Δ5G) was found to be profoundly attenuated in rhesus macaques. Thus, while the acute primary viremia showed viral peaks undistinguishable from those measured in animals infected with the wild-type SIVmac239 infection, viral load during the chronic phase was contained at or below the level of detection [3]. More importantly, these Δ5G "immunized" macaques during the chronic phase manifested near-sterile immunity when challenged with the homologous wild-type SIVmac239, and the animals showed neither evolution of pathogenic revertants nor clinical disease manifestation during a 10 year follow up period. While it is clear that similar live attenuated HIV-1 vaccines will not likely be utilized in humans, it is extremely important to have an animal model that shows protection against heterologous challenge virus so that minimally such a model can be exploited to identify reproducible immune correlates of protection. We therefore reasoned that our SIVmac239-deglycosylation platform may provide an unique opportunity to test and analyze protection against challenge with heterologous isolates.

The studies reported herein utilized a series of four deglycosylated SIVmac239 mutants as potential live attenuated vaccine viruses and the SIVsmE543-3 isolate [4] as the heterologous challenge virus. We submit that the diversities between the vaccine viruses and the challenge virus are equivalent to those found between major HIV-1 subtypes. Thus, this heterologous challenge model provides an ideal model to assess the potential of and define the conditions for cross-subtype (clade) protection against HIV.

The natural protective effects of select rhesus macaque (Mamu) MHC class I alleles such as Mamu B*08, Mamu B*17, Mamu A*01 and the MHC class I haplotype 90120-Ia have been shown to be associated with better control of SIV [5,6,7,8,9]. In sharp contrast, protection by the deglycosylated SIV mutants exhibited no such selectivity; protection was achieved in all 9 rhesus macaques tested so far, which were later found to be indeed genetically highly diverse. Previous human cohort studies revealed that individuals who demonstrated control of HIV infection without any treatment, called long-term non-progressors and elite controllers, have common genetic properties associated with susceptibility to HIV or anti-viral host responses [10,11,12]. However, candidate vaccines that are aimed at targeting outbred human population will have to show effectiveness in humans with diverse genetic backgrounds. In order to minimize the contribution of particular positive or negative genetic background, macaques possessing the above described elite genotypes were therefore eliminated from the studies reported herein. Furthermore, the macaques were grouped based on the genetic data so that each group comprised animals with an essentially similar genetically diverse background.

We herein report data from a series of studies that support the concept that cross-subtype control of HIV-1 is theoretically possible irrespective of genetic background. Data derived herein demonstrate a critical role that glycosylation plays in not only conferring attenuation of SIV/HIV but also the potential role glycosylation plays in conferring pathogenic properties to viruses that emerge following challenge with heterologous viruses.

Results

Genetic diversity of the challenge virus from the vaccine virus

SIVs are as diverse as the HIV-1 subtypes in group M, and at present a total of 9 different SIV lineages have been identified [13]. SIVmac239 belongs to lineage 8. We have generated a variety of modified candidate live vaccine strains by the introduction of deglycosylation mutations into multiple *N*-glycosylation sites of the gp120 of SIVmac239 (Fig. 1). The heterologous challenge virus used in this study is the molecularly cloned pathogenic strain SIVsmE543-3 that belongs to lineage 1. SIVmac239 and SIVsmE543-3 possess 23 and 22 *N*-glycosylation sites, respectively, and as seen their topologies in the gp120 protein backbones are almost the same (Fig. 1).

At first, we compared the amino acid sequence differences for the individual viral proteins between SIVmac239 and SIVsmE543-3 (Table 1). The genetic differences varied from 7.9% for Pol to 35.9% for Tat. We then compared the diversity between the 2 SIV strains utilized herein with the intra-subtype or inter-subtype diversities in the HIV-1 isolates and found that the differences between SIVsmE543-3 and SIVmac239 were significantly greater than any intra-subtype diversities of HIV-1 (Table 1). For the inter-subtype diversity analysis, we used subtypes B and C and a circulating recombinant CRF01_AE as reference strains that are predominantly circulating in Asian countries. The data indicated that the differences between the two SIV strains were as high or higher as those found among the three HIV-1 subtypes. These results validate the use of SIVmac239 as the parental virus for live attenuated vaccine virus and the SIVsmE543-3 as the heterologous challenge virus in the rhesus macaque model of human AIDS.

Properties of the 3 new deglycosylation mutants as live attenuated candidate vaccines

We previously reported that Δ5G, a SIVmac239 molecular clone with quintuple deglycosylation mutations behaved as a live-attenuated virus in vivo [2,3]. In addition to Δ5G, we tested three newly constructed deglycosylated mutants of SIVmac239 viruses, Δ5G-ver1, Δ5G-ver2 and Δ3G as potential candidate vaccines in this study (Fig. 1). They differ by the sites or numbers of N-glycosylation sites mutated in gp120 (Fig. 1). All four deglycosylated mutants replicated well in rhesus peripheral blood mononuclear cells (PBMC) in vitro, and the replication kinetics were similar to SIVmac239 ([2], and data not shown). However, differences were noted in the rate of replication in macrophage cultures and sensitivity to neutralizing antibodies (NAb) (data not shown). To investigate whether these differences translated into altered in vivo properties such as viral replication kinetics in rhesus macaques, reduced pathogenicity and potential vaccine properties, 12 animals were inoculated intravenously in groups of three with 100 $TCID_{50}$ of each of the four mutants (Fig. 2 A). Since the MHC types have been shown to significantly influence the outcome of HIV/SIV infection in their respective hosts, we chose macaques which did not inherit any of the known elite MHC alleles [5,6,7,8,9] (File S1). Furthermore, to minimize the possible influence of other MHC types, we distributed the animals evenly into vaccine and control groups such that each group comprised animals with randomized MHC alleles (File S1).

Consistent with our previous studies [3], the prototypic vaccine strain Δ5G, replicated as robustly as the SIVmac239 in macaques

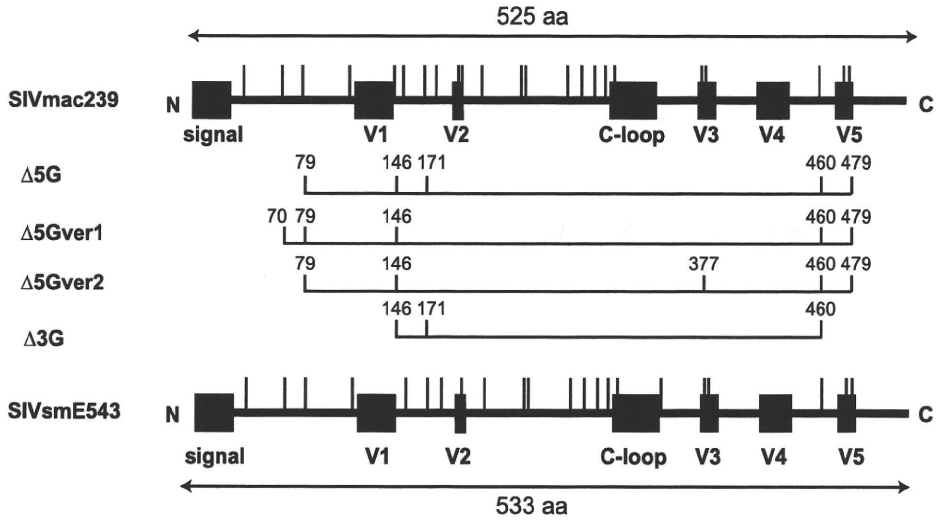

Figure 1. Live attenuated vaccines with deglycosylation mutations. N-glycosylation sites (vertical bars) localized within gp120 of SIVmac239 and SIVsmE543-3 are shown. SIVmac239 and SIVsmE543-3 have 23 and 22 *N*-glycosylation sites, respectively. The position of N-glycosylation sites mutated to remove the specific glycans for Δ5G, Δ5Gver-1, Δ5Gver-2, and Δ3G were indicated and constructed by site-directed mutagenesis based on SIVmac239. V1 to V5 indicate variable region 1 to 5 respectively. C-loop indicates the constant loop region within SIVmac239 [15].
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with peak plasma viral loads (VL) of $\sim 10^7$ copies/ml at 2 weeks post infection (pi) (Figs. 2A and 3). However, subsequently the VL of Δ5G rapidly declined to a level around or below the level of detection (100 copies/ml) whereas relatively high VL persisted in SIVmac239-infected macaques (Figs. 2A and 3). Essentially the kinetics of viremia observed with the three deglycosylation mutants, Δ5G-ver1, Δ5G-ver2 and Δ3G were similar to that seen with Δ5G (Fig. 2 A).

It has been well established that SIVmac239 elicits poor NAb in macaques [14]. In contrast, a deglycosylation mutant derived from SIVmac239 elicited higher NAb than SIVmac239, but levels of NAb responses varied among the animals [15]. Thus, we determined levels of potential NAb responses against each animal's respective infecting virus. Consistent with our previous results, most macaques infected with each of the deglycosylated SIVs induced NAb (Fig. 2 B). However, the levels of NAb responses differed among the four groups, with a decreasing order of magnitude for NAb responses from Δ5G-ver2>Δ5G-ver1>Δ3G>Δ5G. We detected no NAb response in two animals (Mm0301 in the Δ5G group and Mm0304 in the Δ3G group), and delayed and relatively weak responses in three animals (Mm0409 in the Δ5G group, Mm0511 in the Δ5G-ver1 group, and Mm0516 in the Δ3G group) (Fig. 2 B). Regardless of the levels of NAb, all 12 animals infected with the deglycosylation mutant viruses contained

Table 1. Differences between the vaccine and challenge SIV and inter-subtype differences of HIV-1.

Viral proteins	SIV[b]	HIV[a]						
	mac239 vs. smE543-3	Intra-subtype (A, B, C, D, F1, G, CRF01_AE, and CRF_02AG)	Inter-subtype					
			B vs. C		B vs. CRF01		C vs. CRF01	
			Mean	S.E.	Mean	S.E.	Mean	S.E.
Gag	11.1	4.3–7.1	10.2	1.2	11	1.4	12.8	1.5
Pol	7.9	2.8–6.5	7.8	0.7	8	0.8	7.5	0.7
Env	18.2	7.7–12.4	19.2	1.4	18.8	1.4	18.7	1.4
Nef	26.2	9.0–16.2	22.6	4.8	21.3	4.8	16.2	3.5
Tat	35.9	9.9–18.1	28.8	4.7	31.9	4.9	27.4	4.3
Rev	32.7	9.0–16.5	28.3	4.7	26.4	4.7	20.6	4.1
Vif	17.8	7.0–14.2	20.5	2.6	21.6	2.9	21.3	2.8
Vpr	14.9	5.4–10.6	13.1	3.2	13	3.4	5	3.5
Vpx	8.1	NA[c]	NA		NA		NA	
Vpu	NA	2.4–14.8	17.7	5.6	3.8	5.4	12.7	3

[a]Percentage amino acid sequence differences per site from averaging overall sequence pairs between the subtypes.
[b]Percent amino acid sequence differences per protein.
[c]Not applicable.
doi:10.1371/journal.pone.0011678.t001

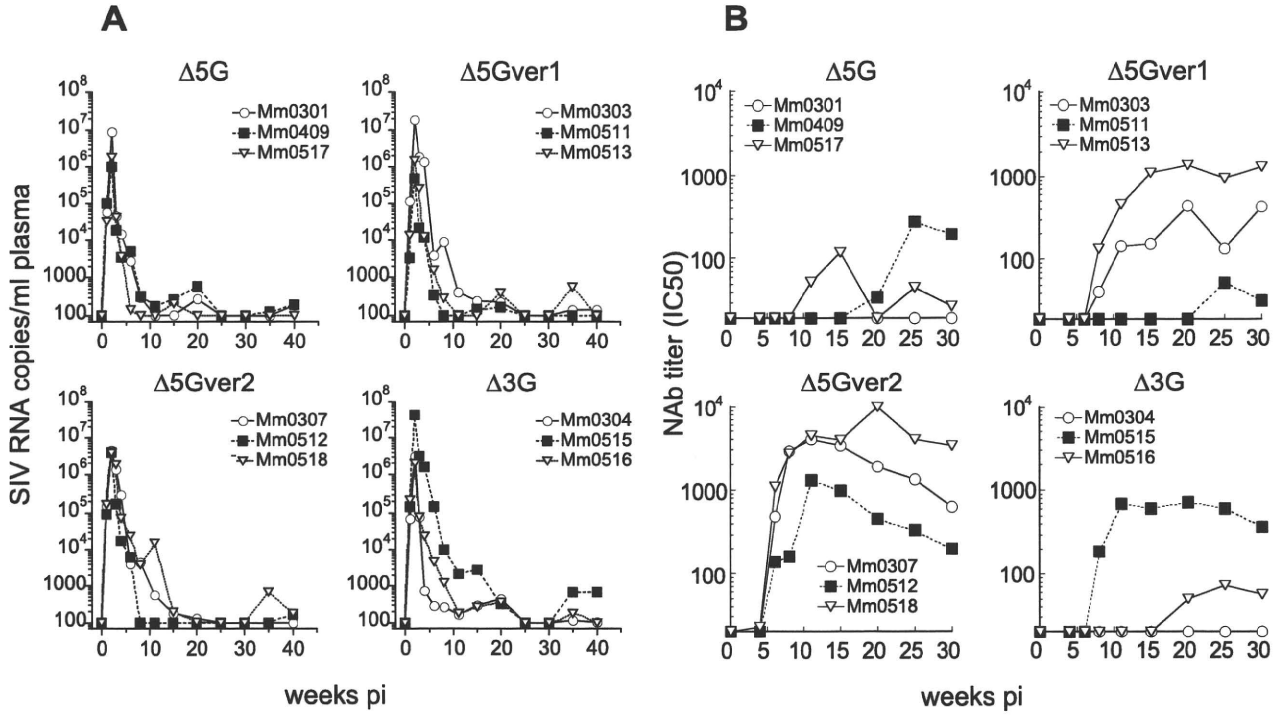

Figure 2. Viral loads and neutralizing antibodies in macaques infected with each of 4 deglycosylated SIV mutants. Twelve animals were divided into 4 groups consist of 3 animals each and infected with each of 4 deglycosylation mutants (Δ5G, Δ5Gver-1, Δ5Gver-2, and Δ3G). (A) Plasma viral loads were determined by real-time RT-PCR with SIVmac239 primers and probe set. (B) NAb responses against each respective infecting virus were measured in CEMx174/SIVLTR-SEAP system. NAb titers were indicated as the reciprocal of the dilutions of the plasma from the vaccinees yielding 50% inhibition (IC_{50}).
doi:10.1371/journal.pone.0011678.g002

primary infection with similar kinetics (Fig. 2 A) suggesting that NAb were most likely not a critical factor for containment of the acute infection in these animals.

We previously found that animals vaccinated with Δ5G completely resist infection when challenged with the parental pathogenic SIVmac239 [3], showing minimal if any replication of the challenge virus for more than 10 years. A similar homologous challenge was performed in a subset of animals that received the deglycosylation mutants in the present study. Thus, one of the three "immunized" animals from each group was challenged with a high dose (1000 $TCID_{50}$) SIVmac239 at 40 weeks following "vaccination" and plasma viral loads were determined (Fig. 3). As previously reported with Δ5G, a near-sterile immunity against challenge with SIVmac239 was not only noted with the Δ5G but also seen with our other three new deglycosylated SIV mutants, Δ5G-ver1, Δ5G-ver2 and Δ3G (Fig. 3). These results indicate that all 3 new vaccine versions possess similar equally high protective potential against the homologous, wild type SIVmac239 as the original Δ5G.

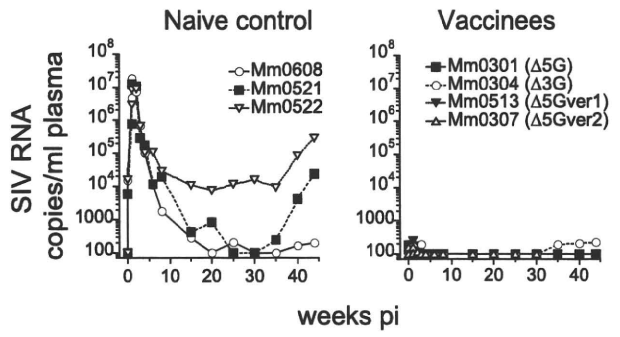

Figure 3. Plasma viral RNA loads in the homologous SIVmac239 challenge. Three naïve rhesus macaques (Mm0608, Mm0521, Mm0522) and 4 vaccinees (Mm0301, Mm0304, Mm0513, Mm0307), i.e. one animal from 4 deglycosylated SIV infection groups, were challenged intravenously with 1000 $TCID_{50}$ of SIVmac239. Plasma viral loads were determined by real-time RT-PCR with SIVmac239 primers and probe set.
doi:10.1371/journal.pone.0011678.g003

Protection of the vaccinated macaques against heterologous challenge infection

The remaining eight animals (2 per group) vaccinated with each of the 4 vaccine versions (Δ5G, Δ5G-ver1, Δ5G-ver2 or Δ3G) and 3 of the four animals that were vaccinated (described in the above paragraph) and challenged with SIVmac239 (Mm0307 died of SIV unrelated causes) were challenged with a high dose (1000 $TCID_{50}$) of SIVsmE543-3 delivered intravenously. Additional three naïve animals served as a control for this heterologous challenge experiment (Fig. 4 A). VL were monitored until 80 weeks post challenge (pc) using real time RT-PCR primer pairs and probes that distinguished the detection of SIVmac239 and SIVsmE543-3.

The 3 naïve control macaques infected with SIVsmE543-3 exhibited a peak VL of $\sim 10^7$ copies/ml at 2 weeks pi which is essentially similar to those we have routinely noted following infection with SIVmac239 with a few exceptions. Notably, the set point VL in SIVsmE543-3 was more than 10^5 copies/ml in 2 animals which is at least 1-log higher than that noted in animals infected with SIVmac239 (Figs. 3 and 4 A). We reason that SIVsmE543-3 is likely to be more pathogenic than SIVmac239 for