Bimodal AIDS Vaccine Approach: Induction of cellular as well as humoral immunity can protect from systemic infection

Robert A. Rasmussen[a,b], **Samir K. Lakhashe**[a,b], and **Ruth M. Ruprecht**[a,b]

[a]Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute, Boston, MA 02115, United States

[b]Harvard Medical School, Boston, MA 02115, United States

Abstract

HIV clade C (HIV-C) strains comprise ~56% of all HIV infections worldwide, and AIDS vaccines intended for global use must protect against this subtype. Our vaccine strategy has been to induce balanced antiviral immunity consisting of both neutralizing antibody and cell-mediated immune responses, an approach we tested in primates. As reported earlier, after isolating recently transmitted HIV-C strains from Zambian infants, we used *env* from one such virus, HIV1084i, to generate a multimeric gp160 immunogen. From another virus, isolated from a different child of the same mother-infant cohort, we cloned *env* to generate a recombinant simian-human immunodeficiency virus (SHIV), which was adapted to rhesus monkeys to yield SHIV-1157ip. Infant macaques were immunized with recombinant viral proteins, including multimeric HIV-C Env 1084i. To test whether cross-protection could be achieved, we mismatched HIV-C Env immunogens and challenge virus *env*. All vaccinated and control monkeys were exposed orally to low-dose SHIV-1157ip. Animals with no or only transient infection were rechallenged intrarectally with a high dose of R5 SHIV-1157ipd3N4, a "late", animal-evolved variant of SHIV-1157ip. Compared to controls, the vaccinees had significantly lower peak viral RNA loads, and one vaccinee remained completely virus-free, even in lymphoid tissues. Data from our novel heterologous mucosal challenge model and our protein-only immunogens imply that significant protection against heterologous viruses circulating in the local community may be achievable with a strategy that seeks to simultaneously induce cellular immunity as well as neutralizing antibody responses.

Keywords

HIV clade C; vaccine; R5 SHIV; animal model; protein immunization; multimeric gp160

*Correspondence to Ruth M. Ruprecht, Department of Cancer, Immunology and AIDS, Dana-Farber Cancer Institute, 44 Binney Street, Boston MA 02115, United States. Tel.: Tel: +1 617 632-3719; fax: +1 617 632-3112. ruth_ruprecht@dfci.harvard.edu (R.M. Ruprecht).

1. Introduction

The development of AIDS vaccines dependent solely upon the induction of cellular immunity has been called into question following the failed STEP human efficacy trial [1]. Those disappointing results supported the general notion that an effective AIDS vaccine will also need to elicit broadly neutralizing antibodies (nAbs). Moreover, the encouraging efficacy data from the recently completed Phase III vaccine trial in Thailand, whereby a combination of cellular immunity-inducing canarypox-based ALVAC vCP1521 plus antibody-inducing AIDSVAX B/E resulted in 31% protection, provides additional new data suggesting that an efficacious vaccine should generate a balanced immune response of both cellular and humoral antiviral immunity [http://news.bbc.co.uk/2/hi/health/8272113.stm].

The simian-human immunodeficiency virus (SHIV)/rhesus macaque model has provided proof-of principle that nAbs alone – given as passive immunization – can protect against immunodeficiency virus infection [reviewed in 2]. To test the efficacy of a vaccine designed to induce both cellular and humoral immunity, the virus challenge should approximate real-life conditions that mimic the most important aspects of HIV transmission among humans [3]. Some of these conditions would include repeated mucosal exposure with an R5 virus that is non-homologous to the strain of virus(es) used for vaccine construction. Until recently, a mucosally transmissible R5 SHIV clade C (SHIV-C) had been unavailable to test vaccine efficacy against HIV clade C (HIV-C). Here, we used two SHIV-C variants, SHIV-1157ip and SHIV-1157ipd3N4 [4], viruses that originated from SHIV-1157i, a chimera containing the SHIV-vpu$^+$ backbone (expressing simian immunodeficiency virus (SIV) mac239 *gag* and *nef* [5]) and incorporating most of gp120 plus the entire extracellular and transmembrane domains of gp41 of HIV1157i, a recently transmitted virus isolated from a Zambian infant. SHIV-1157ip, the early biological isolate, was obtained after passage through 5 rhesus macaques during peak viremia, an adaptation strategy we devised to avoid selecting neutralization escape viruses. Three of the five monkeys developed AIDS [6], and a late virus, SHIV-1157ipd, was isolated from one of them. A molecular clone was derived using the 5' half of SHIV-vpu$^+$ and the 3' half of SHIV-1157ipd with an extra NF-κB binding site in the long terminal repeats. The resulting SHIV-1157ipd3N4 is highly replication competent and exclusively R5-tropic.

We developed these SHIV-C strains to evaluate candidate vaccines for use in infants against mucosal HIV-C transmission. Earlier, we showed strong containment of SHIV clade B (SHIV-B) replication in macaques immunized as infants solely with multimeric clade B gp160 [7–9]. In the studies described here, we incorporated multimeric clade C gp160 into a protein vaccine that also contained SIV Gag-Pol particles and HIV Tat. We deliberately mismatched SHIV-C *env* and the Env immunogen, which was derived from HIV1084i [10] isolated from a postnatally infected infant who was part of the same patient cohort at Lusaka Hospital as infant 1157i, the source of SHIV-C *env*. We reasoned that primate vaccine efficacy testing should approximate the real-life situation where human AIDS vaccine recipients will likely encounter HIV strains differing from those used to generate vaccines, although they may be exposed to strains circulating in the local community [3].

Materials and methods

2.1 Animals

Indian-origin rhesus monkeys (*Macaca mulatta*) were housed at the Yerkes National Primate Research Center (YNPRC), Atlanta, GA, a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All procedures were approved by the Animal Care and Use Committees of Emory University and the Dana-Farber Cancer Institute.

2.2 Immunogens

The pJW-gp160–1084i DNA plasmid encoding *env* derived from the pediatric isolate, HIV1084i [10], has been described [11]. Codon-optimized gene fragments of SIVmac239 *gag-pro* (1.8 kb) and SHIV89.6P *tat* (0.3 kb) were PCR amplified from multiple annealed overlapping oligonucleotide primers (100 bp each) [12]. Each fragment was cloned into the mammalian expression plasmid pJW4303 [13]. Animals receiving DNA were inoculated intradermally with 250 μg of each plasmid (total 750 μg DNA) at each time point; control animals (Groups 3 and 3a) received 750 μg of empty pJW4303 DNA plasmid vector. For protein inoculations, HIV-C gp160 was prepared from recombinant vaccinia virus infected BSC-40 cells as described previously [14]. SIV Gag-Pol particles were prepared as essentially as described [15–17] using an early-late synthetic promoter and HIV Tat was purchased from Advanced Bioscience Laboratories, Inc. (Kensington, MD). Proteins were given i.m. in incomplete Freund's adjuvant at 100 μg of each per inoculation.

2.3 Humoral immune responses

ELISAs for anti-Env antibody binding were performed as described [13,18]. Antibody titers were calculated as reciprocal serum dilution giving O.D. readings >5 standard deviations above background as calculated using prebleed serum at the same dilutions. Neutralizing antibody titers were measured using a viral infectivity assay of TZM-bl cells (obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, National Institute of Allergy and Infectious Disease, National Institute of Health, Bethesda MD) as described previously [13,18]. Due to the limitations in the amount of blood that could be collected from infant macaques, preimmune sera were not always available. As such, percent neutralization was calculated relative to luciferase activity level in negative control serum samples consisting of virus + pooled sera from 5 naive rhesus macaques to offset potential virus proliferation inhibitors/and or enhancers occasionally present in macaque serum. Neutralizing antibody titers were estimated as the reciprocal serum dilution giving 50% inhibition of virus replication.

2.4 Virus-specific cellular immunity

PBMC were isolated from whole blood by centrifugation in heparinized CPT tubes (BD Biosciences-Pharmigen) and washed 3× prior to assay. For antigen-specific T-cell stimulation, overlapping 20-mer SIVmac239 Gag and 15-mer HIV Tat peptides representing the complete protein sequences were obtained through ARRRP. Interferon (IFN)-γ ELISPOT analysis was done as described [18] using plates coated with purified mouse anti-

human IFN-γ (BD Biosciences-Pharmigen, San Diego, CA). Cells were incubated overnight at 10^5 cells/well in assay medium plus pooled peptides of SIVmac239 Gag (pools #1, #2 and #3 consisting of peptides 1–42, 43–84 and 85–125, respectively) or HIV Tat. Assays were done in duplicate and background levels were determined by incubation of cells without peptides. Intracellular staining after peptide stimulation was performed essentially as described elsewhere [19].

2.5 Viruses and challenges

SHIV-1157ip and SHIV-1157ipd3N4 have been described [4]; the amino acid Env sequences of both viruses share 93.2% homology (Fig 1). The amino acid sequence of the HIV1084i gp160 used for immunization shares 78.8 and 78.4% homology with SHIV-1157ip Env and SHIV-1157ipd3N4 Env, respectively (Fig. 1). Amino acid homology was determined using the 'SIM-Alignment Tool for protein sequences' at http://expasy.org/tools/sim-prot.html utilizing the Blosum 62 Comparison matrix with a gap open penalty of 12 and a gap extension penalty of 4. Virus stocks of each were prepared from concanavalin A-stimulated naive rhesus PBMC; TNF-α (10 ng/ml) was included for generating the SHIV-1157ipd3N4 stock only.

2.6 Viral load and statistical measurements

Plasma vRNA loads were assessed by quantitative RT-PCR for SIV *gag* sequences (QiaAmp RNA Blood Mini-Kit – Qiagen) [20] Peak viremia levels were compared by 2-sided Wilcoxon rank-sum test.

3. Results

3.1 Vaccination and pre-challenge immune responses

Five macaques were given the multigenic protein-only vaccine (Group 1, Fig. 2), Group 2 (5 animals) received a more conventional bimodal DNA prime/protein boost regimen, and Group 3 (controls) received empty vector DNA. DNA vaccination consisting of codon-optimized plasmids encoding SIV *gag-protease*, HIV *tat* and heterologous HIV1084i clade C *env* started on postnatal d 1–5 (Group 2, Fig. 2); another DNA immunization was given at week 6. Group 2 was rested and then given soluble protein boosts consisting of SIV Gag-Pol particles [15–17], HIV Tat and HIV1084i gp160. At this time, age-matched Group 1 animals were vaccinated with the viral proteins.

Two weeks after the final protein inoculation, all groups were challenged orally with heterologous SHIV-1157ip. At this time, IFN-γ ELISPOT analysis of peripheral blood mononuclear cells (PBMC) (Table 1) showed strong SIV Gag-specific reactivity in three Group 1 animals (RLu-9, RAt-9 and RSr-9); RSr-9 also had strong HIV Tat-specific ELISPOT responses. Two animals from Group 2 (RHy-9 and RRz-9) demonstrated strong SIV Gag-specific and HIV Tat-specific ELISPOT responses. No specific reactivity was observed in any controls (Group 3). All 10 animals from Groups 1 and 2 had high-titer binding antibodies to homologous HIV1084i gp160, a relatively neutralization resistant virus (Table 1). Only one animal of Group 1 had measurable anti-HIV1084i nAb activity. Importantly, 9 of 10 vaccinees had significant nAb activity to the heterologous challenge

virus, SHIV-1157ip. Controls (Group 3) showed no anti-Env-C antibodies. These data demonstrate that multimeric HIV-C gp160 (as part of a multigenic protein vaccine) or DNA priming/protein boosting induced cross-reactive nAb to SHIV-C in macaques.

3.2 Low-dose oral challenge with early SHIV-1157ip

Oral SHIV-1157ip challenge employed a relatively low dose (3.7 50% animal infectious doses (AID_{50}); (Fig. 3A and B) in an attempt to approximate the lower HIV-C inocula that are likely involved in milk-borne HIV-C transmission [21]. Three animals from Group 1 (RAt-9, RSr-9 and RNu-9) remained virus-free and 2 animals from Group 2 (RJy-9 and RBa-10) were only transiently viremic with viral RNA (vRNA) loads of 1,465 and 267 vRNA copies/ml of plasma, respectively (Fig. 3A). At this virus dose, 4 of 5 controls became systemically infected (Fig. 3B).

3.3 High-dose intrarectal challenge with late SHIV-1157ipd3N4

Next, we sought to test whether vaccinees with no or transient infection after low-dose challenge might be protected from high-dose challenge with the more virulent SHIV-1157ipd3N4. Thus, animals that remained uninfected or had <1,000 vRNA copies/ml plasma were rechallenged intrarectally with SHIV-1157ipd3N4. This included 3 animals from Group 1 (RAt-9, RSr-9 and RNu-9), one animal from Group 2 (RBa-10), and one from control Group 3 (RAr-9) (Fig. 3C and D). After rechallenge with SHIV-1157ipd3N4, the non-vaccinated animal, RAr-9, had a peak plasma viremia level of 7.7×10^7 vRNA copies/ml (Fig. 3D); similarly, eight additional age-matched, non-vaccinated controls that received the same dose of SHIV-1175ipd3N4 intrarectally (Group 3a, Fig. 1) had peak plasma viremia levels ranging from $1.3 – 7.1 \times 10^7$ vRNA copies/ml (Fig. 3D). All controls, i.e. animals of Group 3a plus RAr-9, rapidly became viremic with vRNA loads ranging from $10^3 – 10^5$ copies/ml at 1 week post-exposure. In contrast, only 1 of 4 previously vaccinated monkeys had detectable virus by week 1 after SHIV-1157ipd3N4 exposure. Among the vaccinees that became infected, peak plasma viremia levels ranged from $3.3 – 7.4 \times 10^6$ copies/ml, i.e. a log lower than controls (Fig. 3C; p=0.003, rechallenged vaccinees versus controls). Importantly, one vaccinee of Group 1 given protein only, RAt-9, had no evidence of infection after rechallenge with high-dose SHIV-1157ipd3N4. RNA isolated from a peripheral lymph node biopsy of RAt-9 at week 10 post SHIV-1157ipd3N4 rechallenge was also negative by RT-PCR analysis. These results indicate that protein immunization alone can confer complete protection against a sequential low-dose/high-dose mucosal challenge with heterologous R5 SHIV-C.

We then sought to delineate the immune parameters that may explain the complete protection of RAt-9 at the time of SHIV-1157ipd3N4 rechallenge. At this time, RAt-9 had much higher virus-specific cellular immune reactivity to both SIV Gag (8,615 spot-forming units (SFU)/10^6 PBMC for all 3 Gag peptide pools) and HIV Tat (705 SFU/10^6 PBMC) than the rechallenged vaccinees that had evidence of viral containment, RBa-10 and RNu-9 (240 and 550 anti-Gag IFN-γ ELISPOTs, respectively, and none against Tat). Of note, control monkey RAr-9 that had been exposed orally to SHIV-1157ip without becoming infected had no specific antiviral cellular immunity at the time of rechallenge. We then compared total ELISPOT reactivity against all Gag plus Tat peptide pools at the time of the first and second

challenges (Table 2). The protected animal, RAt-9, was the only one with a large increase of total ELISPOT activity. Even the transiently infected animal, RBa-10, had fewer total ELISPOTs at the time of the second challenge compared to the first. Thus, protection in RAt-9 was associated with a strong boosting effect via the initial virus exposure.

Although the protected animal, RAt-9, had a nAb titer of 1:160 against the first virus, SHIV-1157ip (Table 2), nAb activity against the rechallenge virus was minimal, reflecting the relative neutralization resistance of the late virus, SHIV-1157ipd3N4. However, RAt-9 was the only double-challenged animal that maintained nAb titers during the 6 months between challenges. NAb activity of all the other rechallenged animals was minimal or negative against all viruses tested. These data suggest that virus-specific T-cell immunity was associated with complete protection of RAt-9 from mucosal SHIV-1157ipd3N4 challenge as only low-level nAb activity against the 2nd challenge virus was seen.

3.4 Long-lived immunity in the protected animal, RAt-9

Repeated attempts to detect plasma viremia in the protected animal, RAt-9, were negative. In addition, transfer of blood from RAt-9 to infant macaques that were depleted of CD8+ cells by anti-CD8 mAb treatment also failed to reveal any virus replication that may have been contained by virus-specific immune responses in RAt-9 (data not shown).

A long-term follow up immune assessment of animal RAt-9 revealed that it maintained high nAb levels against the early virus, SHIV-1157ip as late as two years after challenge with the second virus (Table 3). At no time did RAt-9 generate more than minimal nAb activity against the second challenge virus, SHIV-1157ipd3N4, a more difficult to neutralize, tier 2 variant. Gag-specific cellular immune responses in PBMC, as measured by IFN-γ ELISPOT and intracellular cytokine staining analysis also demonstrated that RAt-9 maintained long-lived virus-specific cellular immunity.

4. Discussion

The course of the protected monkey, RAt-9, sheds light onto the mechanisms responsible for immune protection. The clear-cut boosting of cellular antiviral immunity leads us to postulate that this animal had sub-threshold infection with the live early virus, which was reined in by pre-existing antiviral immunity and thus never became overt. This nevertheless led to strong amplification of cellular immunity and the maintenance of nAb titers against the early virus. When this animal then encountered the high dose of the relatively neutralization-resistant late virus, cellular immunity – probably supported by antibody-dependent cellular cytotoxicity (ADCC), which was demonstrated to be high at the time of challenge (data not shown) – then held SHIV-1157ipd3N4 in check. Of note, this protected animal continues to maintain very high levels of antivirus ELISPOT activity more than 3 years after challenge.

The protection from the initial low-dose SHIV-1157ip challenge in animals immunized solely with soluble viral proteins, including multimeric Env, is consistent with our previous study showing containment of SHIV-B in macaques immunized with multimeric Env only [7–9]; high nAb activity was associated with, but not predictive of virus containment.

Several groups have pursued AIDS vaccine strategies based upon multimeric gp140 to maintain conserved, conformational Env determinants in an effort to generate broadly reactive nAbs [22–28]. Our data show that multimeric gp160 derived from a recently transmitted HIV clade C strain is a potent immunogen and can generate protective antiviral immunity when used as part of a more complex protein vaccine. The fact that we observed either viral containment or complete protection is promising, especially when considering the single high-dose (20 AID$_{50}$) intrarectal challenge with the late virus.

The strain of HIV any individual may be exposed to will be heterologous to the strain (or strains) used for developing the vaccine components. We propose that AIDS vaccines, especially those seeking to induce broad-based humoral immunity, should be tested against SHIV strains that are heterologous to the HIV Env component of the vaccine. The degree of strain diversity for such testing is shown schematically in Fig. 4 as a series of ever expanding concentric circles. In this schema, the diversity between the Envs in the vaccine and the challenge SHIV increases progressively. The smallest degree of Env divergence is represented by intra-clade strains isolated within a local community – as in our case – then expands to regionally divergent intra-clade strains to global intraclade strain differences; cross-clade diversity between the vaccine Env and SHIV challenge Env represents the greatest divergence. Our upfront heterologous challenge study, based upon locally divergent HIV-C *env* genes, was designed to model repeated human mucosal exposure to evolving strains circulating within the community. This is represented as the second tier of Env diversity between vaccine strain and challenge strain (Fig. 4) and, indeed, vaccination with multimeric gp160 from a recently transmitted local strain was able to induce nAbs against a heterologous challenge virus.

Acknowledgments

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                          ←----- signal peptide -----→           → start of gp120
                                       20         30                  50        60        70
                      ....|....| ....|....|  ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        MRVRGIQRNY PQW-WIWG--  ILGFLMIYNG MGSLWVTVYY GVPVWKEAKT TLFCASDAKA YEREVHNIWA
SHIV-1157ip gp160     MRVKEKYQHL WRWGWRWGTM  LLGMLMICSA TEKLWVTVYY GVPVWKEAKT TLFCASDAKA YEKEVHNIWA
SHIV-1157ipd3N4 gp160 MRVKEKYQHL WRWGWRWGIM  LLGMLMICSA TEKLWVTVYY GVPVWKEAKT TLFCASNAKA YEKEVHNIWA

                               80        90       100       110       120       130    ←-- V1 --
                                                                                            0
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        THACVPTDPN PQELVLENVT ENFNMWKNDM VDQMHEDIIS LWDQSLKPCV KLTPLCVTLN CTDVKSANST
SHIV-1157ip gp160     THACVPTDPN PQEIVLENVT ENFNMWKDDM VDQMHEDIIS LWDQSLKPCV KLTPLCVTLK CSNFTREGNV
SHIV-1157ipd3N4 gp160 THACVPTDPN PQEIVLGNVT ENFNMWKNDM VDQMHEDIIS LWDQSLKPCV KLTSLCVTLK CSNFTGKSNV

                           150       160       170       V2    180       190       200       210
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        S-----EDMR NCSFNVTTER KDRKKLEQAL FYRLDIVPLK NSSSSNFSEY RLINCNTSTV SQACPKVNFD
SHIV-1157ip gp160     TYKEEMDKVK NCSFNVTTGI RDKKQKVNAL FYRLDITPLD ENNN-NSSEY RLINCNSSTI TQACPKVNFD
SHIV-1157ipd3N4 gp160 TYKGDME-VK NCSFNVTTEI RDKKQKVYAL FYRLDITPLD DN----SSEY ILINCNSSTI TQACPKVNFD

                           220       230       240       250       260       270       280
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        PIPIHYCAPA GYAILKCNNK TFNGSGPCNN VSTVQCTHGI KPVVSTQLLL NGSLAEEDII IRSENLTNNV
SHIV-1157ip gp160     PIPIHYCAPA GYAILKCNNK TFNGTGPCHN VSTVQCTHGI KPVVSTQLLL NGSLAEREII IRSENLTDNV
SHIV-1157ipd3N4 gp160 PIPIHYCAPA GYAILKCNNK TFNGTGPCHN VSTVQCTHGI KPVVSTQLLL NGSLAEGEII IRSENLTDNV

                           290       300       310       V3    320         330     340       350
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        KTIIVHLKDY VEIVCTRPNN NTRKSMRIGP GQAFYATGEI IGNIREAHCN ISGSKWNNTL QRVKKKLGEH
SHIV-1157ip gp160     KTIIVHFNES VEINCTRPNN NTRKSIRIGP GQAFYATGDI IGDIRQAHCN ISKENWNKTL QWVRGKLEEH
SHIV-1157ipd3N4 gp160 KTIIVHFNES VEITCTRPNN NTRKSISIGP GQAIYATGDI IGDIRQAHCN ISKENWNKTL QWVRGKLKEH

                           360       370       380       390       V4 400        410     420
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        FPNNTTIDFK PSSGGDLEIT THSFNCRGEF FYCNTSKLFN GTS------ ESNSTITLPC KIKQIINMWQ
SHIV-1157ip gp160     FPN-KTIVFK PSSGGDLEIT THSFNCRGEF FYCNTSKLFN GTDNSTHMDT GNDTVITIPC RINQIINMWQ
SHIV-1157ipd3N4 gp160 FPN-KTIVFK PSSGGDLEIT THSFNCRGEF FYCNTSKLFN STDNSTHMGT ENNTIITIPC RIKQIINMWQ

                           430       440       450       ←-- V5 460      470       480       490
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        GVGRAMYAPP IAGNITCKSN ITGLLLTCKSN IFREGGGDMR DNWRSELYKY KVVKIEPLGI
SHIV-1157ip gp160     EVGRAMYAPP IEGNITCKSN ITGLLLVRDG GQDNSTNNTE TFREGGGDMR NNWRSELYKY KVVEIKPLGI
SHIV-1157ipd3N4 gp160 EVGRAMYAPP IEGNITCKSN ITGLLLVRDG GWDNSTNDTE TFREGGGDMR DNWRSELYKY KVVEVKPLGI

                           500       start gp41/fusion peptide      530       540       550  ←-- N-term-
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        APTKAKRRVV ERGKRAVGIG ALFLGFLGAA GSTMGAASLT LTVQARQLLS GIVQQQNNLL RAIEAQQHML
SHIV-1157ip gp160     APTKAKRRVV EREKRAVGIG AVFLGFLGAA GSTMGAASIT LTVQARQLLS GIVQQQDNLL RAIEAQQHML
SHIV-1157ipd3N4 gp160 APTKAKRRVV EREKRAVGIG AVFLGFLGAA GSTMGAASIT LTVQARQLLS GIVQQQDNLL RAIEAQQHML

                      inal heptad repeat region →         590       600       610       620       630
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        QLTVWGIKQL QARVLAIERY LQDQQLLGIW GCSGKLICTT DVPWNSSWSS KSYEDIWTNM TWMQWDKEIN
SHIV-1157ip gp160     QLTVWGIKQL QARVLAIERY LQDQQLLGIW GCSGKLICTT AVPWNASWSN KSQTDIWENM TWMQWDKEIS
SHIV-1157ipd3N4 gp160 QLTVWGIKQL QARVLAIERY LQDQQLLGIW GCSGKLICTT AVPWNASWSN KSQTDIWENM TWMQWDREIS

                      ←------ C-terminal heptad repeat region ------→ 670       ← MSD1 →  ← MSD2 → 00
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        NYTNTIYQLL VDSQTQQEKN ERELLALDSW KNLWNWFNIT NWLWYIKIFI MIVGGLIGLR IIFAVLSMVN
SHIV-1157ip gp160     KHTDTIYRLL EDSQNQQEKN EKDLLALDSW ENLWNWFSIT KWLWYIKIFI MIVGGLMGLR IIFAVLSIVS
SHIV-1157ipd3N4 gp160 RHTDTIYRLL EDSQNQQEKN EKDLLALDSW KNLWNWFSIT RWLWYIKIFI MIVGGLIGLR IIFAVLSIVN

                      ← MSD3 →             intracellular
                           710       720       730       740                               770
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        RVRQGYSPLS FQTLTPNPRG PDRLGRIEEE GGEQDRDRSI RLVSGFLALA WDDLRSLCLF SYHRLRDCIL
SHIV-1157ip gp160     RVRQGYSPLS FQTHLPTPRG PDRPEGIEEE GGERDRDRSI RLVNGSLALI WDDLRSLCLF SYHRLRDLLL
SHIV-1157ipd3N4 gp160 RVRQGYSPLS FQTHLPLPRG ADRPEGIEEE GGERDRDRSI RLVTGSLALI WDDLRSLCLF SYHRLRDLLL

                           780       790       800       810       820       830       840
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        IVARAAELLG RSSLRGLQKG WEALKYLGSL VQYWGLELKK SAISLLDTTA IAVAEG---- ----------
SHIV-1157ip gp160     IVTRIVELLG R-------RG WEALKYWWNL LQYWSQELKN SAVSLLNATA IAVAEGTDRV IEVVQYGWSY
SHIV-1157ipd3N4 gp160 IVTRTVELLG R-------RG WEALKYWWNL LLYWSQELKN SAVSLLNATA IAVR------ ----QYGWSY

                           850       860       870       880       890
                      ....|....| ....|....| ....|....| ....|....| ....|....|
HIV1048i gp160        ---------- ---------T DRIIKLIQRI CRAICNIPRR IRQGFEAALQ
SHIV-1157ip gp160     FHEAVQAVWR FATETLAGAW GDLWETLRGG GRWILAIPRR IRQGLELTLL
SHIV-1157ipd3N4 gp160 FHEAVQAVWR SATETLAGAW GDLWEILRRG GRWILAIPRR IRQGLELTLL
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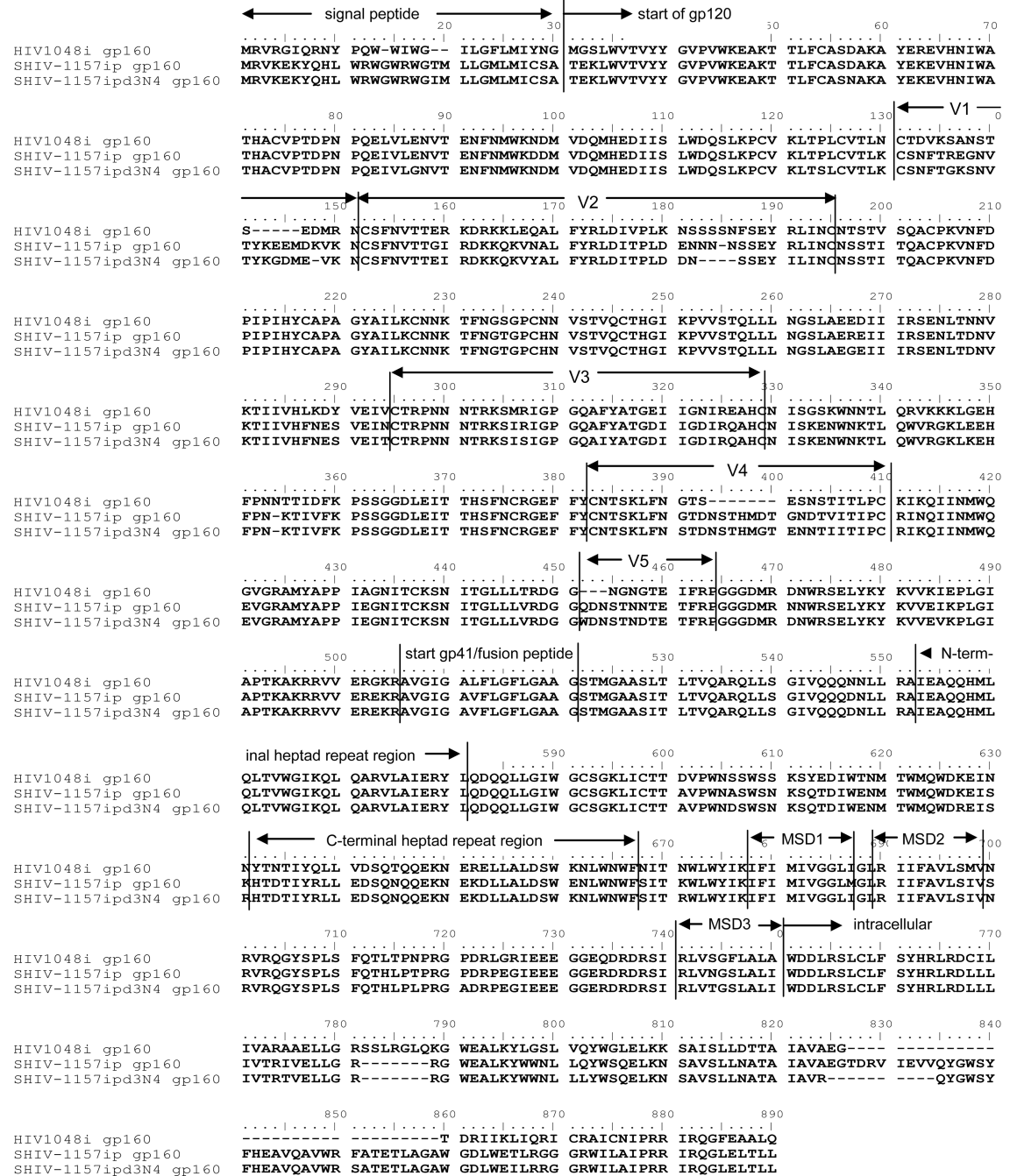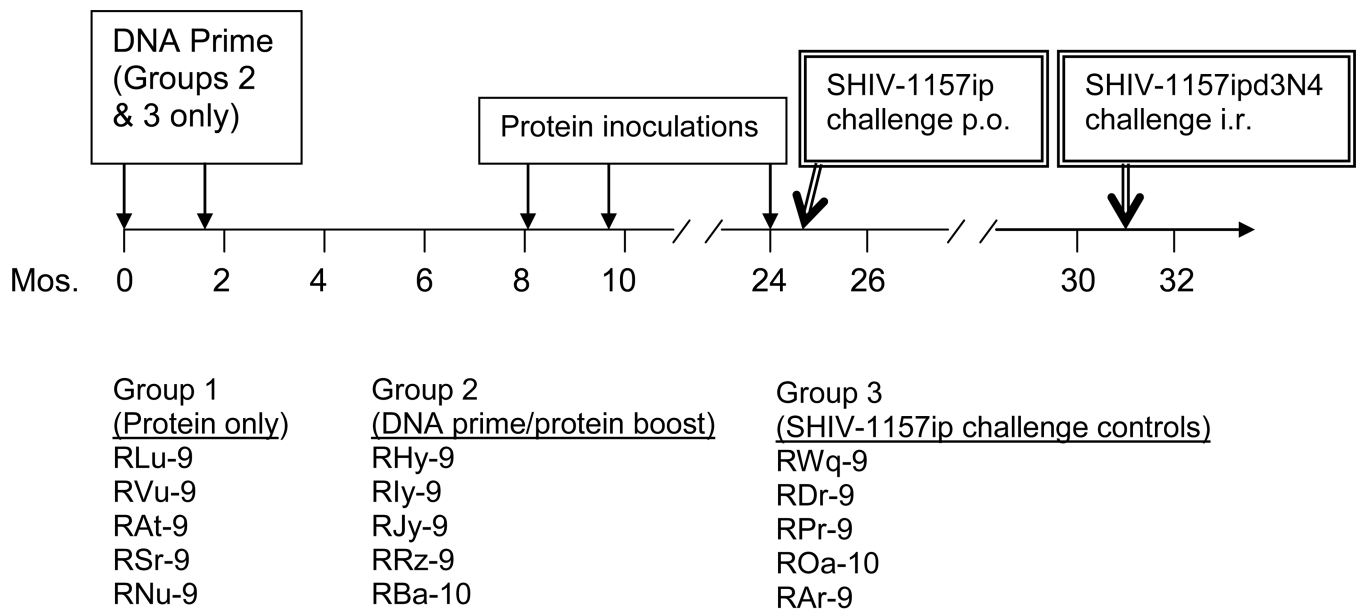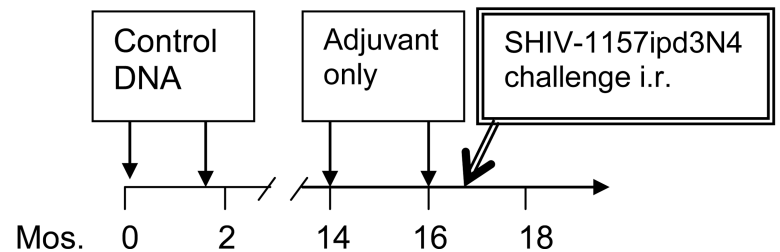
Fig. 1. Amino acid sequence alignment of HIV1084i, SHIV-1157ip and SHIV-1157ipd3N4 Envs
MSD: membrane spanning domain.

**Fig. 2. Protein or DNA prime/protein boost vaccination and low-dose/high-dose virus challenge schema in infant macaques**

Animals of Groups 2 and 3 were inoculated with DNA starting between d1–5 of birth. Animals of Group 2 received DNA encoding SIV *gag-pro*, HIV *tat* and HIV1084i gp160 *env,* while Group 3 animals were given control DNA. Protein immunizations, consisting of SIV Gag-Pol particles, HIV Tat and multimeric HIV1084i gp160, were initiated at mo 8 and given to Group 2 animals and age-matched, naïve Group 1 animals. Two weeks after the 3rd protein inoculation of Group 1 and 2 animals, all monkeys were challenged orally with SHIV-1157ip (3.7 50% animal infectious doses (AID$_{50}$)). Animals with no or only transient infection, as described in the text, were rechallenged 6 mo later with a high dose of SHIV-1157ipd3N4 (20 AID$_{50}$, intrarectally).
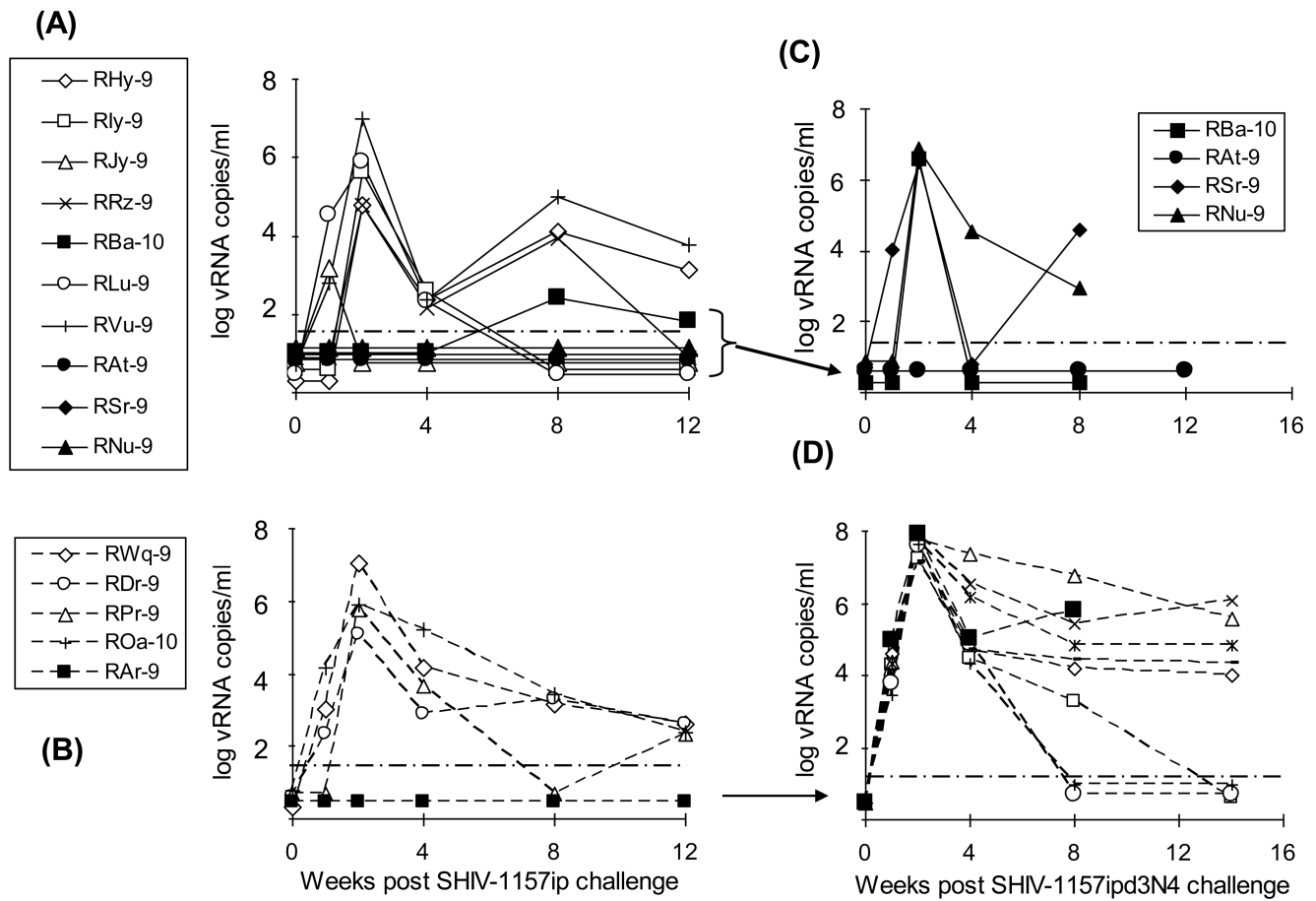
Fig. 3. Plasma viremia post-SHIV-1157ip and SHIV-1157ipd3N4 challenges
Viral loads after oral challenge with SHIV-1157ip in plasma of animals of Groups 1 and 2
(A) and 3 (B) and after intrarectal rechallenge with SHIV-1157ipd3N4 of animals of Groups
1 and 2 (C) and 3 (D); viral loads of 8 additional SHIV-1157ipd3N4 challenged control
animals are also shown (D). Solid symbols in (A) show viremia levels in those animals of
Groups 1 and 2 that were later rechallenged with SHIV-1157ipd3N4. Solid square symbols
in (B) and (D) show viremia levels in Group 3 control animal, RAr-9, after each challenge,
respectively. The solid circle symbols in (C) highlight undetectable viremia levels of animal
RAt-9 after rechallenge. The assay's limit of detection (50 copies/ml plasma) is denoted by
dashed, horizontal lines (adapted from [13]).
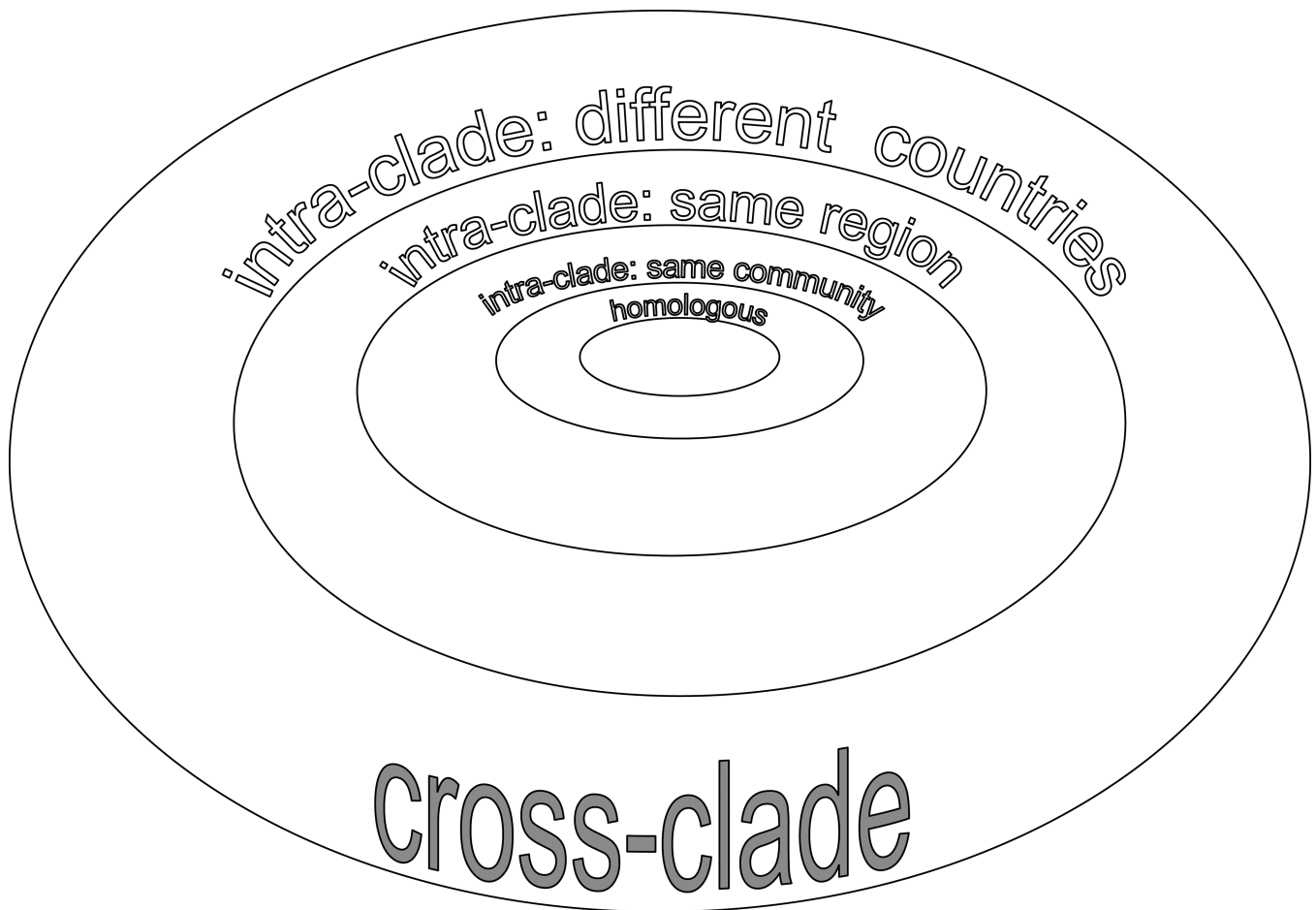
Fig 4. Increasing the diversity between immunogens and Env in challenge SHIV strains
The degree of heterogeneity between the strain of Env in a potential vaccine and the
challenge strain of virus is shown schematically as increasingly larger concentric circles.

Table 1

Prechallenge immune responses in rhesus macaques.

		T cell reactivity[1]		HIV1084i gp160 Ab binding titers	nAb titers[2]	
		SIV Gag	HIV Tat		HIV1084i	SHIV-1157ip
Group 1 (protein only)	RLu-9	4,500	310	3.1×10^5	20	130
	RNu-9	370	70	3.1×10^4	<10	<10
	RVu-9	20	180	6.2×10^5	<10	15
	RSr-9	4,400	1,560	6.2×10^5	<10	160
	RAt-9	3,790	40	6.2×10^4	<10	130
Group 2 (DNA prime/protein boost)	RHy-9	7,320	3,600	3.1×10^5	<10	50
	RIy-9	270	140	6.2×10^5	<10	20
	RJy-9	130	20	3.1×10^4	<10	140
	RRz-9	7,130	3,100	3.1×10^4	<10	640
	RBa-10	370	450	6.2×10^4	<10	120
Group 3 (control)	RWq-9	0	0	$<10^3$	<10	<10
	RAr-9	0	0	$<10^3$	<10	<10
	RDr-9	40	0	$<10^3$	<10	<10
	RPr-9	20	0	$<10^3$	<10	<10
	ROa-10	0	0	$<10^3$	<10	<10

PBMC and sera of individual animals were tested 2 weeks after the final protein inoculations (day of SHIV-1157ip challenge).

[1] T-cell responses in PBMC to peptide pools of the indicated viral antigen were determined by IFN-γ ELISPOT analysis and given as SFU/10^6 PBMC. Responses to each of three SIV Gag peptide pools have been summed.

[2] Serum neutralizing antibody titers to HIV1084i and SHIV-1157ip were measured using the TZM-bl-based reporter assay and show the reciprocal dilution of sera giving 50% neutralization. adapted from Rasmussen et al.[13]

Table 2

Immune parameters and viral outcome of both SHIV challenges in rechallenged RM[1].

| | total ELISPOTS[2] | | SHIV-1157ip nAb activity | | |
	SFU	fold change from 1st challenge	titer	fold change from 1st challenge[3]	vRNA
RAt-9					
1st challenge	3,830		130		<50
2nd challenge	9,320	2.4×	160	1.2×	<50
RSr-9					
1st challenge	5,960		160		<50
2nd challenge	160	0.007	<10	0.06	3.3×10^6
RNu-9					
1st challenge	440		<10		<50
2nd challenge	550	1.25×	<10	-	7.4×10^6
RBa-10					
1st challenge	820		120		transient – 267
2nd challenge	240	0.29	<10	0.08	3.8×10^6
RAr-9 (Control)					
1st challenge	0		<10		<50
2nd challenge	5	-	<10	-	7.7×10^7

[1] Comparison of virus-specific ELISPOT reactivity, SHIV-1157ip nAb activity and peak plasma viremia levels at the time of 1st challenge (SHIV-1157ip) and 2nd challenge (SHIV-1157ipd3N4) from animals that were rechallenged with SHIV-1157ipd3N4. Values for the completely protected animal, RAt-9, are underlined.

[2] The sum of all HIV-Tat and SIV Gag-specific ELISPOTS at the time of each challenge.

[3] Where 1st challenge values were <10, the magnitude fold-change was calculated using a value of 9. adapted from Rasmussen et al. [13]

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Table 3

Long-term immune reactivity of protected animal, RAt-9.

NAb reactivity:

time post-SHIV-1157ipd3N4 challenge	SHIV-1157ip	SHIV-1157ipd3N4
18 mos	90	<10
24 mos	110	<10

Cellular immune reactivity[1]:

SIV Gag-specific ELISPOTS:	$5,853 \pm 910/10^6$ PBMC		
Intracellular staining:	SIV Gag peptide stimulation		
	% IFN-γ	%TNF-α	% IL-2
CD3+CD8+	0.38	1.14	0.58
CD3+CD4+	0.92	2.61	2.61

[1] Cellular immune reactivity was tested 41 months post-SHIV-1157ipd3N4 challenge. Intracellular staining values show the percentage of each gated population showing expression of the indicated cytokine. Background values (no stimulation) have been subtracted.