HIV-1 recombinant poxvirus vaccine induces cross-protection against HIV-2 challenge in rhesus macaques

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Rhesus macaques were immunized with attenuated vaccinia or canarypox human immunodeficiency virus type 1 (HIV-1) recombinants and boosted with HIV-1 protein subunits formulated in alum. Following challenge with HIV-$2_{SBL6669}$, three out of eight immunized macaques resisted infection for six months and another exhibited significantly delayed infection, whereas all three naive controls became infected. Immunizations elicited both humoral and cellular immune responses; however, no clear correlates of protection were discerned. Although more extensive studies are now called for, this first demonstration of cross-protection between HIV-1 and -2 suggests that viral variability may not be an insurmountable problem in the design of a global AIDS vaccine.

One of the main problems hindering HIV-1 vaccine development is the extensive variability of the virus. HIV-1 variants not only are observed within and between infected individuals, but worldwide, eight distinct subtypes, A through H, have been described[1]. A ninth grouping, subtype O, is composed of even more highly divergent members. As a result of this heterogeneity, design of an effective vaccine for even a geographically restricted area is a daunting task. The problem is compounded on a global scale, where tailoring of vaccines to specific populations is a current strategy.

Although HIV-2 is clearly related to HIV-1 structurally and functionally, it nevertheless constitutes a different viral type. The viruses exhibit limited homology, with only 40 to 60% similarity in the amino acid sequences of the structural gene products[2]. Although less pathogenic and infectious than HIV-1, HIV-2 also causes AIDS, albeit with a slower disease course[3–7]. HIV-2 is endemic in West Africa, and has become a growing public health problem. Therefore, our AIDS vaccine studies have addressed not only HIV-1 but also HIV-2, in part as a model for HIV-1, but also as a legitimate candidate itself for vaccine development.

Our investigations have focussed on attenuated poxvirus recombinants as vaccine vehicles. NYVAC, derived from the Copenhagen vaccinia virus vaccine strain, was attenuated by the precise deletion of 18 open reading frames implicated in poxvirus virulence[8,9]. The naturally attenuated ALVAC is a canary poxvirus vector system that is unable to productively replicate in non-avian species[8,9]. Despite their highly attenuated phenotype, NYVAC and ALVAC have been shown to function effectively as immunization vehicles[8–10] (unpublished results). With respect to the use of these vectors in retrovirus vaccine development, an ALVAC recombinant expressing the feline leukaemia virus (A subtype) Env and Gag proteins was shown to afford protection in cats against the development of persistent viraemia when challenged with virus simultaneously given in the mouth and sprayed on the nose[11]. Moreover, protocols consisting of an ALVAC-HIV-1_{MN} gp160 recombinant followed by boosting with native envelope protein (gp120) demonstrated the ability of the ALVAC-HIV-1 recombinant to prime HIV-1 specific humoral and cellular immune responses in Phase I human trials, even in individuals previously exposed to vaccinia (J.-L. Excler, oral presentation, Septième colloque des "Cent Gardes", 1992).

Lack of an affordable, readily available, and proven animal model for HIV-1 has greatly hindered vaccine research. The chimpanzee is inappropriate on the first two counts, and the pig-tailed macaque (*Macaca nemestrina*) model[12], although promising, has yet to be proven reliable. For these reasons we have chosen to use the rhesus macaque (*M. mulatta*) system. This species has been shown to be susceptible to infection not only by several simian immunodeficiency virus (SIV) strains but also by HIV-2 (ref. 13–16), thus allowing not only assessment of HIV-2 vaccine efficacy, but also evaluation of HIV-1 vaccine immunogenicity followed by heterologous cross-challenge experiments.

In separate studies a lasting protection against HIV-2 infection of rhesus macaques (G. Franchini *et al.*, manuscript submitted) and human T-cell leukaemia/lymphoma virus type I

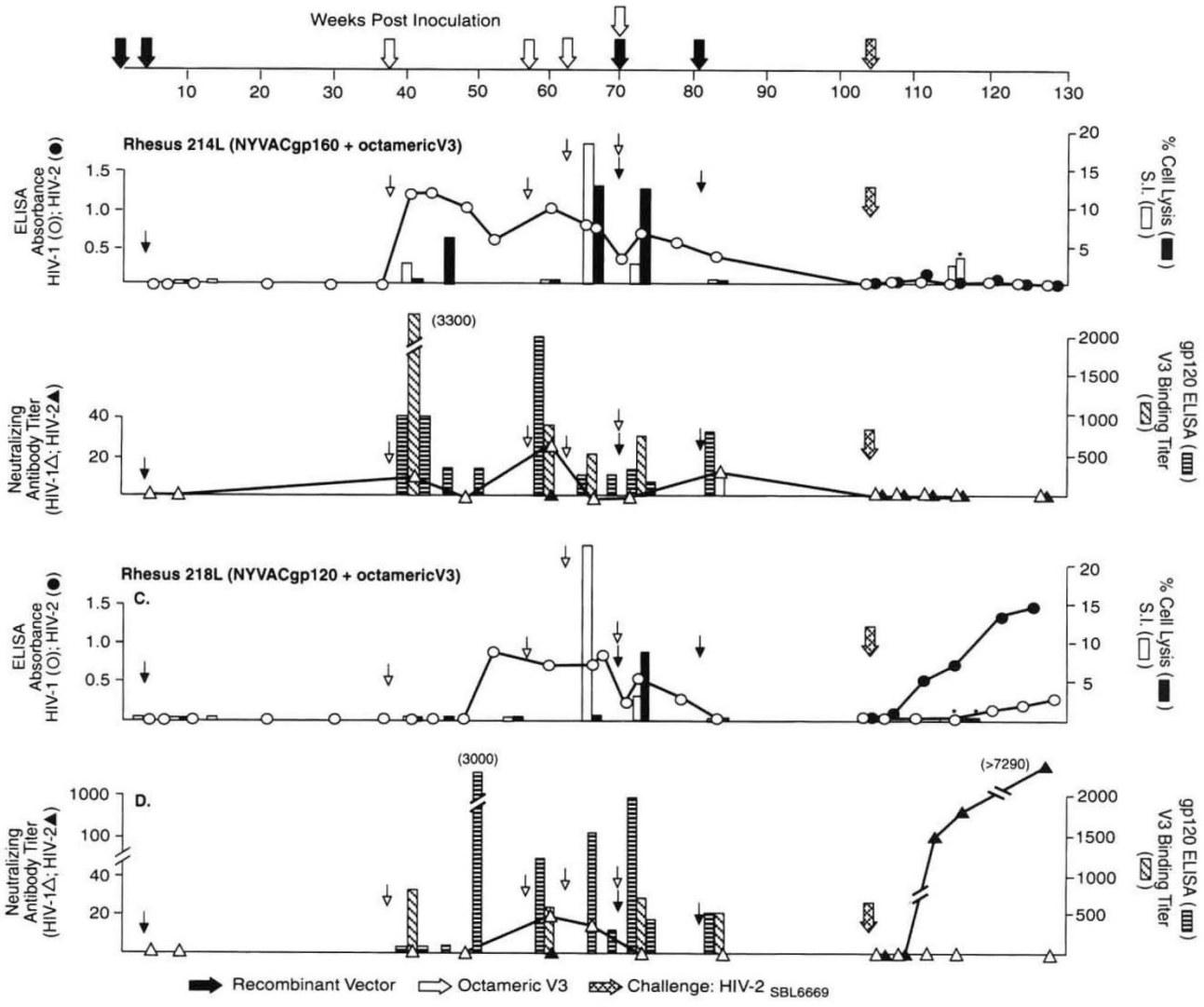

Fig. 1 Immune responses in rhesus macaques inoculated with NYVAC-HIV-1_{IIIB} gp160 or gp120, and boosted with octameric V3 peptide. Macaque 214L (top two panels) was immunized (closed arrows) with the NYVAC-HIV-1_{IIIB} gp160 recombinant, vP911, and macaque 218L (lower two panels) with the NYVAC-HIV-1_{IIIB} gp120 recombinant vP921. Both macaques were boosted (open arrows) with a mixed octameric V3 loop peptide preparation. The final boost with NYVAC recombinants consisted of 10^8 pfu for macaque 214L and 10^9 pfu for macaque 218L. cell-mediated immune responses marked with an asterisk (*) were obtained with HIV-2 antigens and targets. The time of challenge with HIV-$2_{SBL6669}$ is indicated by the large hatched arrow.

(HTLV-I) infection of rabbits[17] has been demonstrated following immunization with HIV-2 or HTLV-I recombinant vaccine candidates constructed in either NYVAC or ALVAC vectors. To pursue these promising vectors as HIV-1 candidate vaccines, we first evaluated the immunogenicity of a number of NYVAC and ALVAC recombinants carrying HIV-1 genes (*gag, pol, env*) followed by various viral proteins or peptides presented with adjuvants in eight rhesus macaques. Our objectives were (1) to compare the priming ability of recombinants expressing both HIV-1 Env proteins (gp120, gp41) versus simply the gp120 moiety alone; (2) to evaluate recombinants carrying the full complement of HIV-1 genes for viral structural proteins *gag, pol,* and *env*, as well as those carrying just the *env* gene; (3) to compare directly NYVAC- and ALVAC-HIV-1 recombinants in similar prime/ boost protocols; and (4) to compare these results with those obtained using NYVAC- and ALVAC-HIV-2 recombinants and proteins for vaccinating rhesus macaques against HIV-2 (G. Franchini *et al.*, manuscript submitted). At the conclusion of the immunogenicity studies, we evaluated the protection afforded by the immunization regimens against a heterologous cross challenge with HIV-2.

As expected our results showed that the NYVAC and ALVAC recombinants primed both humoral and cellular immune responses to HIV-1. But remarkably, following live virus challenge 50% of the immunized animals resisted infection by HIV-2, while all three naive controls became infected. This finding, although preliminary, suggests that viral variability may not necessarily preclude effective AIDS vaccine development. It should encourage further investigation of cross-protection in other heterologous systems as well as stimulate research on immunologically conserved epitopes and potentially novel protective mechanisms.

Results

Immunogenicity of HIV-1 recombinants and boosts

Eight rhesus macaques were inoculated with NYVAC- or ALVAC-HIV-1 recombinants and subsequently boosted with peptides or HIV-1 proteins formulated in alum or with additional ALVAC/NYVAC-HIV-1 recombinants (see Methods). Macaques were bled at intervals and sera were assayed for antibodies to the entire HIV-1 envelope protein and the V3 loop, a principal neutralizing determinant of the virus[18–21], and for neutralizing antibodies. Peripheral blood mononuclear cells (PBMC) were monitored for HIV-1-specific lymphocyte proliferative responses as well as cytolytic activities. The HIV-specific immune responses observed are in Figs 1–4.

A comparison of NYVAC recombinants carrying either the gp160 or gp120 gene showed that each was equally capable of priming immune responses in rhesus macaques (Figs 1 & 2). A booster inoculation with viral protein subunit preparations was required to elicit detectable humoral and cellular immune responses, the latter directed primarily to the V3 loop epitope. With the exception of animal 219L, which exhibited neutralizing antibodies following a single subunit booster inoculation (Fig. 2), two subunit booster inoculations were necessary to elicit neutralizing antibody activity and also led to more regularly detectable Env-specific T-cell proliferative responses and cytolytic activities. Additional booster inoculations, either with subunit preparations or recombinants, had little effect on subsequent immune responses. Animals boosted with native gp120 (Fig. 2) developed higher neutralizing antibody titres compared to those boosted with the octameric V3 peptide (Fig. 1), although the latter macaques exhibited higher V3 loop binding titres, suggesting that native gp120 elicited neutralizing antibodies to epitopes in addition to the V3 loop. This is further indicated by the independent fluctuation of the gp120 and V3 loop binding titres in animals 215L and 219L, and the closer correlation of neutralizing antibody titre with gp120 binding titre compared to V3 loop binding titre (Fig. 2).

The NYVAC- and ALVAC-HIV-1 recombinants carrying *gag* and *pol* genes, in addition to *env*, also primed both humoral and cellular immune responses, and these responses appeared earlier than those elicited by the NYVAC-*env* recombinants. V3 binding antibodies, consisting of both IgM and IgG iso-

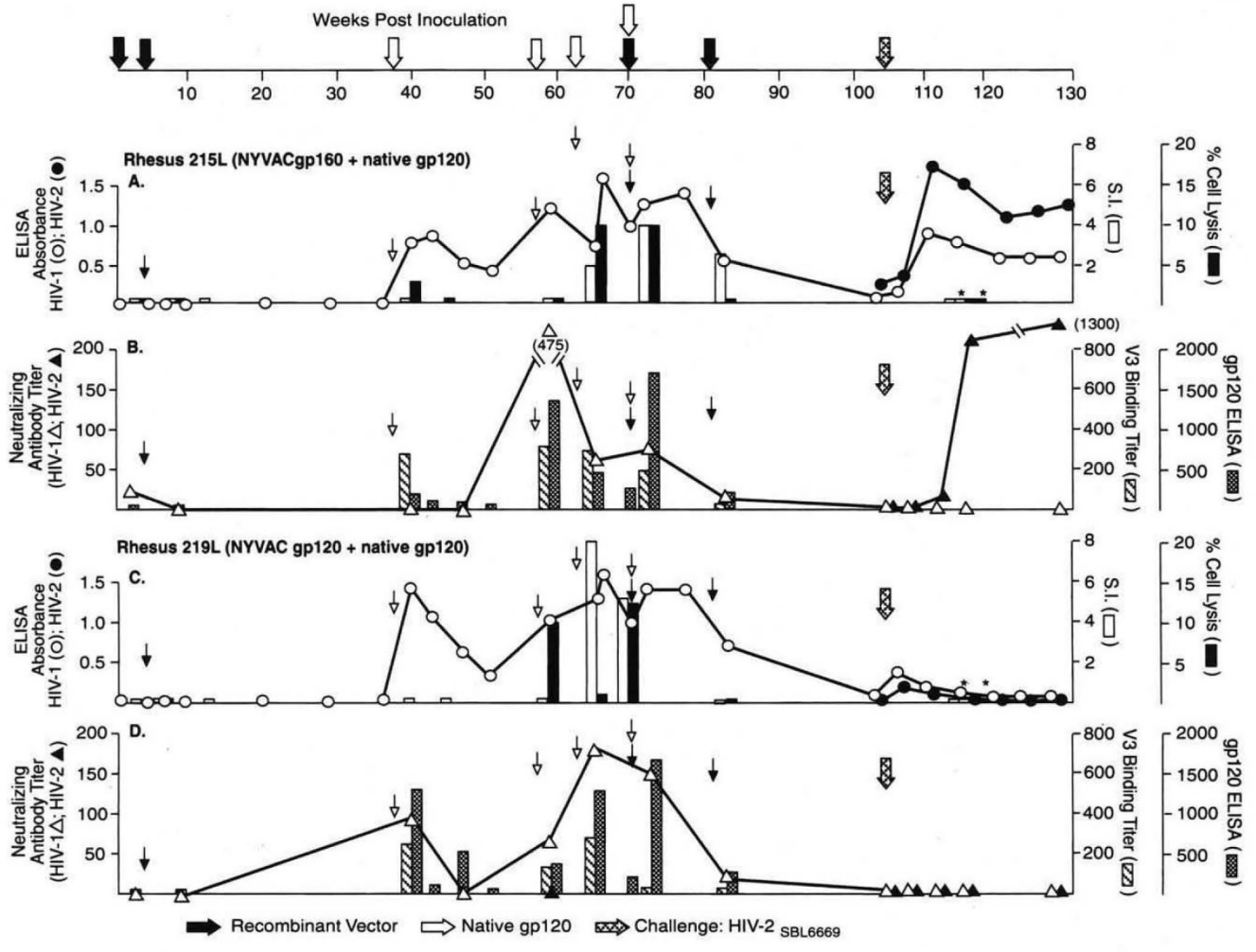

Fig. 2 Immune responses in rhesus macaques inoculated with NYVAC-HIV-1_{IIIB} gp160 or gp120 and boosted with native gp120. Macaque 215L (top two panels) and 219L (lower two panels) were immunized with NYVAC-HIV-1_{IIIB} gp160 (vP911) or gp120 (vP921), respectively, and boosted with native HIV-1_{IIIB} gp120. The final recombinant boost for 215L consisted of 10^8 pfu of vP911 and for 219L, 10^9 pfu of vP921.

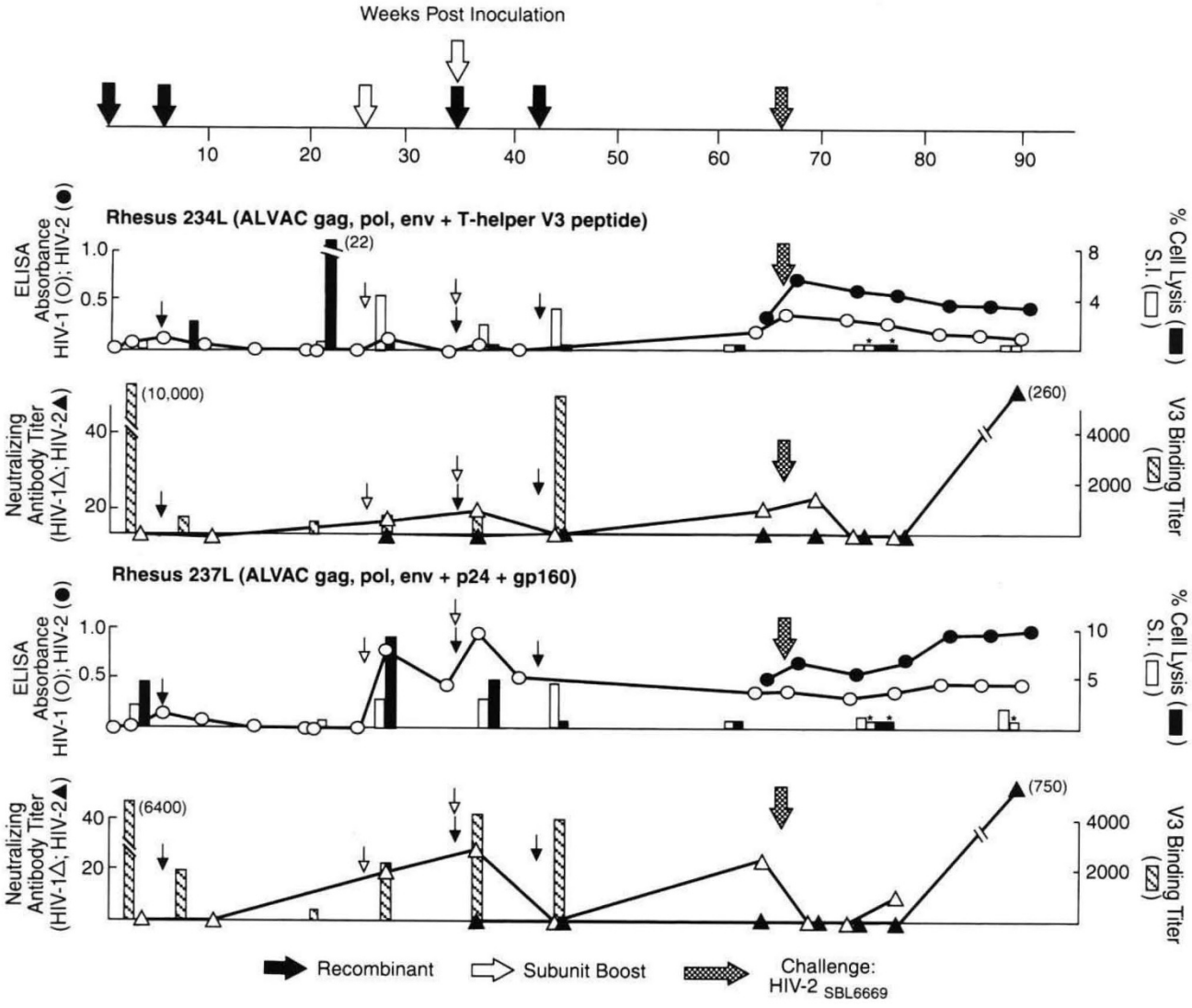

Fig. 3 Immune responses in rhesus macaques inoculated with ALVAC-HIV-1_{MN} *gag, pol, env,* and boosted with p24 plus gp160 or T-helper-V3 peptide. Macaques 234L and 237L received a mixture of four ALVAC-HIV-1_{MN} recombinants (see Methods). The final boost with recombinants consisted of 10^8 pfu of each for both macaques 234L and 237L. Subunit boosts consisted of either native p24 and gp160 (MN/LAI chimera) for macaque 237L or a tandem T-helper-V3 peptide for macaque 234L.

types, were evident following the first recombinant immunization (Figs 3 and 4). In addition, low-level cellular immune responses to the V3 loop, envelope helper epitopes, and the HGP-30 peptide were detectable following 1 or 2 recombinant immunizations (Figs 3 and 4). Again, two booster inoculations with viral protein subunits were required to elicit peak neutralizing antibody titres, but subsequent booster inoculations had no appreciable effect on either humoral or cellular immune responses. As expected, boosting with native proteins elicited higher anti-envelope antibody responses than did the tandem T-helper-V3 peptide, although similar V3 binding titres were achieved with both immunogens. The highest neutralizing antibody titre achieved by any of the eight immunized macaques appeared in macaque 238L (Fig. 4), immunized with NYVAC *gag, pol* and *env,* and boosted with the p24 T-helper-MN V3 peptide, although this result was not reproduced in the paired macaque (234L) primed with ALVAC *gag, pol* and *env* (Fig. 3). Levels of other immune responses elicited in the macaques immunized with the mixed NYVAC or ALVAC recombinants were comparable. It should be noted that the low ELISA reactivities before challenge in Figs 3 and 4 most likely reflect assay of sera of macaques immunized with HIV-1_{MN} recombinants and subunits on IIIB gp120 envelope protein.

Challenge with HIV-2

Five months following the last boost with HIV-1 recombinant, the eight immunized macaques and three naive controls (J198, J190 and L1) were challenged with HIV-$2_{SBL6669}$ grown in macaque PBMC. All the control animals, shared with a parallel study (G. Franchini *et al.,* manuscript submitted) seroconverted, and HIV-2 could be detected in their PBMC by polymerase chain reaction (PCR) and/or virus isolation within 2 months of infection (Table 1). Of the eight vaccinated macaques, three (214L, 219L and 239L) were completely protected from viral infection as judged by the in-

ability to isolate virus from or detect viral sequences by nested PCR in macaque PBMC during the 6 months following challenge. One other animal (234L) exhibited partial protection manifested by the inability to isolate virus from or detect viral sequences in PBMC during the 6 months following challenge; however, neutralizing antibody appeared 6 months post-challenge (Table 1, Fig. 3). Four of the vaccinated macaques became infected. It was possible to repeatedly isolate virus from the PBMC of three of these macaques (215L, 218L and 238L) beginning 2–6 weeks post-challenge. Moreover, HIV-2-specific neutralizing antibodies rapidly increased to high titres in these animals following challenge (Figs 1, 2 and 4). Macaque 237L exhibited slightly delayed infection (Table 1) and like the partially protected 234L exhibited low titre HIV-2-specific neutralizing antibody only at 6 months post-challenge (Fig. 3). The three fully protected macaques (214L, 219L and 239L) failed to develop such antibodies, and in fact exhibited no anamnestic response post-challenge at all (Figs 1, 2 and 4). In comparison, in the infected control animals, the appearance of neutralizing antibodies paralleled the detection of virus. By 6 months post-challenge the control macaques exhibited neutralizing antibody titres of 565 (L1), 165 (J190) and 655 (J198). These results suggest that the development of neutralizing antibodies to the challenge virus is a highly sensitive indicator of viral infection as observed also in the HIV-2 macaque (G. Franchini *et al.*, manuscript submitted) and HTLV-I rabbit studies[17]. Cell-mediated immunity in response to HIV-2 antigens was not observed in the vaccinated but infected macaques after challenge except for macaque 238L, which exhibited low HIV-2-specific cytotoxic activity (Fig. 4).

Discussion

The pilot studies described here were designed to evaluate the immunogenicity of several HIV-1 recombinants and the possibility of cross-protection from a heterologous challenge virus. With regard to the first objective, immunization with either NYVAC- or ALVAC-HIV-1 recombinants followed by booster

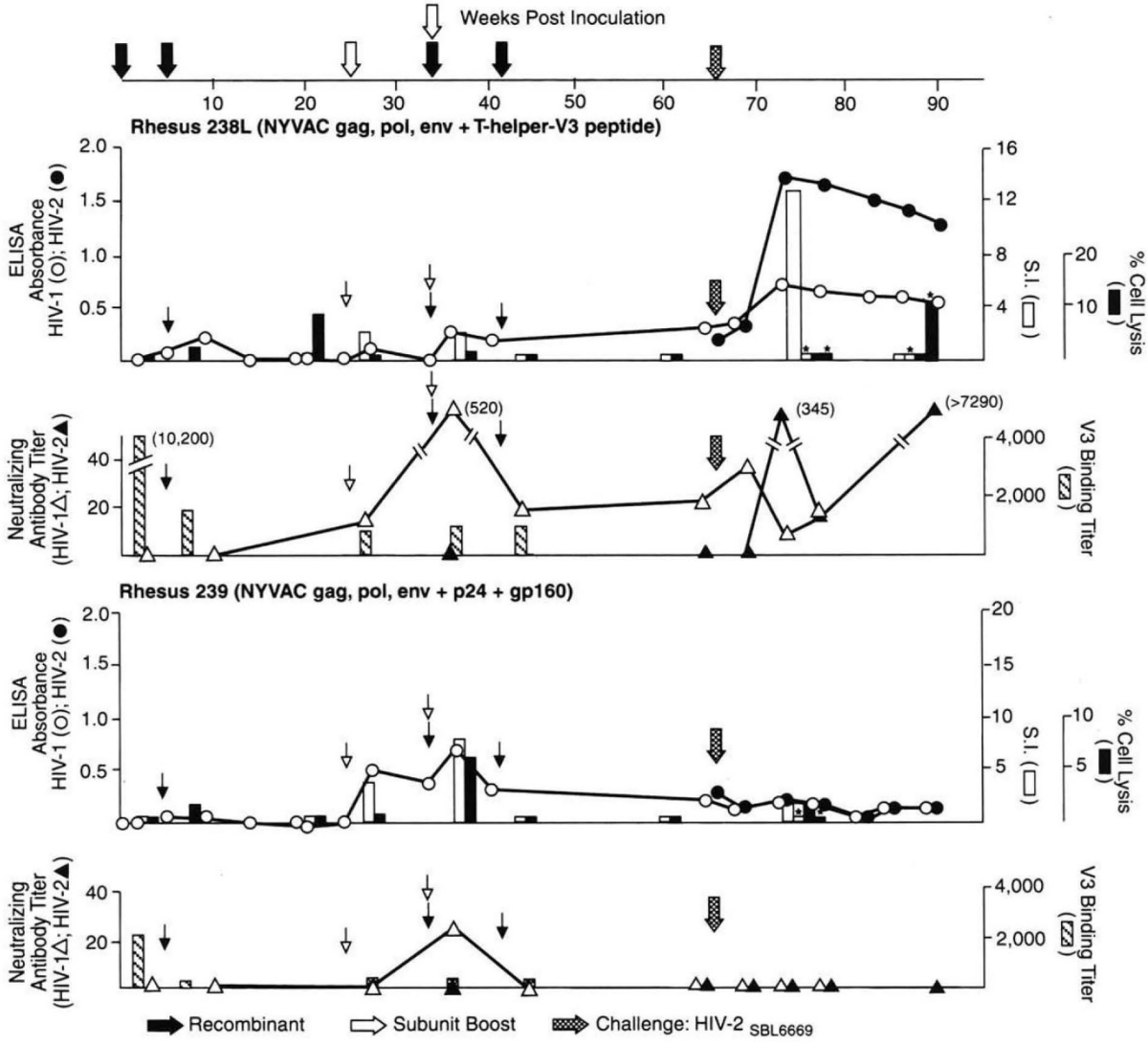

Fig. 4 Immune responses in rhesus macaques inoculated with NYVAC-HIV-1_{MN} *gag, pol, env,* and boosted with p24 plus gp160 or with the T-helper V3 peptide. A mixture of NYVAC HIV-1_{MN} recombinants was used to immunize macaques 238L and 239L. Macaque 238L was boosted with the tandem T-helper V3 peptide and macaque 239L with p24 plus chimeric gp160. The final boost consisted of 10^8 pfu of each recombinant.

inoculations with HIV-1 protein subunits elicited good humoral and cellular immune responses. Recombinants containing *gag, pol* and *env* genes elicited earlier responses compared to those containing *env* alone, perhaps due to better antigen presentation. The structural proteins expressed by poxvirus recombinants carrying *gag, pol* and *env* gene products form particles *in vitro* (unpublished observations), which presumably are efficiently taken up by antigen-presenting cells and processed. A strict comparison of responses elicited by recombinants carrying only the *env* gene with those of recombinants carrying *gag, pol* and *env* is not possible, as the former were HIV-1_{IIIB} constructs and the latter HIV-1_{MN}. Moreover, immunization with the mixed recombinants was carried out with a total dose of 4×10^7 pfu, whereas only 10^7 pfu were used in priming the macaques with the single env gene recombinants.

The requirement for subunit booster inoculations for elicitation of significant HIV-specific immune responses in this HIV-1 rhesus macaque system is in contrast to results obtained using NYVAC- and ALVAC-HIV-2 recombinants, in which cellular immunity was evident following immunization with recombinant viruses alone (G. Franchini *et al.*, manuscript submitted). This disparity may reflect a basic difference in immunogenicity between the HIV-1 and HIV-2 recombinants used. In both studies, strong humoral immunity required subunit boosters, although a stronger boosting effect was observed in the HIV-1 system described here compared to the HIV-2 system. It is difficult to attribute the difference either to the priming vectors or the subunit boosts. The subunits used here were either native proteins or synthetic peptides, while in the HIV-2 study gp160 produced in insect cells was used. With regard to subunit booster inoculations in general, it is notable that in a vaccine study using attenuated poxvirus HTLV-I recombinants, a protein booster inoculation, while eliciting an immune response, appeared to be deleterious with respect to protection from subsequent viral challenge[17].

Table 1 Summary of viral detection in immunized and control rhesus macaques following challenge with HIV-$2_{SBL6669}$

		Months post-HIV-2 challenge						
	Viral detection							Protective Status*
Macaques Immunized		1	2	3	4	5	6	
214L	VI	-	-		-	-	-	P
	PCR	-	-			-		
215L	VI	+	+		+	-		I
	PCR	+	+			+		
218L	VI	-	+		+	+		I
	PCR	-	+			+		
219L	VI	-	-		-	-	-	P
	PCR	-	-			-		
234L	VI	-	-	-	-	-	-	PP
	PCR	-	-			-		
237L	VI	-	-	+	-	-	-	I
	PCR	-	-	+		+		
238L	VI	+	+	+	-			I
	PCR	+	+	+		+		
239L	VI	-	-	-	-	-	-	P
	PCR	-	-			-		
Controls								
LI	VI	-	-		+			P
	PCR	-	+					
JI90	VI	+						I
	PCR	+	+					
JI98	VI	+			+			I
	PCR	+	+					

*P, protected. PP, partially protected. I, infected. VI, virus isolated. PCR, viral genome detected by PCR.

The basis for the cross-protection obtained in four (three protected, one partially protected) of the eight vaccinated macaques is unknown. No clear immune correlates of protection were observed (Table 2), similar to results of other vaccine studies in macaques[22–24]. Before challenge, immunized macaques demonstrated sporadic HIV-1-specific cell-mediated immune responses and good neutralizing antibody activity against HIV-1, with titres as high as 520. However, no cross-neutralizing antibodies against the HIV-2 challenge virus were present before or at the time of challenge exposure. HIV-2 cross-reactive cytotoxic T lymphocytes (CTL) were not measured. Following challenge, HIV-1 specific humoral and cellular immune responses were recalled primarily in macaques immunized with constructs containing *env, gag* and *pol* genes suggesting shared epitopes between the two viruses (Figs 3 and 4). Two of the macaques that became infected (215L and 238L) exhibited the highest neutralizing titres against HIV-1 before challenge. Although these high titres had declined by the time of challenge, the question of possible cross-reactive enhancing antibodies remains to be explored. In general, both protected and infected immunized macaques exhibited modest levels of humoral and cellular immunity before challenge.

Whether conserved epitopes shared between the distantly related HIV-1 and HIV-2 isolates are immunogenic in rhesus macaques and could have contributed to the protection observed must be determined by further study. Common antigenic determinants and cross-reactive epitopes between HIV-1 and HIV-2, including cross-neutralizing antibodies[25–28], and cross-reactive CTL[29,30] have been described, the latter recently implicated in protection of women from repeated HIV exposure[30]. However, the sporadic detection of HIV-specific T-cell proliferative and cytolytic responses in PBMC did not suggest a strong role for cellular immunity in the protection obtained. We have observed, however, that cell-mediated immune responses can sometimes be more reliably detected and robust in tissues of macaques[31] suggesting that responses seen in the periphery may not reflect the true cellular immune state of the animal. Other protective mechanisms were considered, such as antibody-dependent cellular cytotoxicity (ADCC). Sera of HIV-1-infected patients have been shown to elicit ADCC against HIV-1 and HIV-2-infected cells[32]. However, failure to identify such activity in sera of our protected macaques (not shown) suggests that this immune response did not participate in the protection obtained. In the absence of a firm immunological basis for the observed protection, the possibility that protection resulted from a novel mechanism, for ex-

ample induction of a cytokine or inhibitory factor, should be considered in future studies.

In this study, three of six macaques immunized with NYVAC recombinants (both NYVAC-*env* and NYVAC-*env, gag, pol*) were fully protected, regardless of the boosting schedule, whereas one macaque that received the ALVAC construct carrying all three structural genes was partially protected. However, these numbers are too few to draw any conclusions regarding the better construct.

In summary, at the very least, a prolonged and marked suppression of replication of an infectious swarm of HIV-$2_{SBL6669}$ was observed following immunization of rhesus macaques with NYVAC- or ALVAC-HIV-1 constructs and boosting with HIV-1 protein or peptides. These results provide the first demonstration of cross-protection between highly divergent HIVs, suggesting that broad vaccine protection may be achievable. The exciting possibility that immunization with HIV-1 vaccines might give the added bonus of protection against HIV-2 infection would greatly facilitate vaccination programs in regions such as West Africa, where both HIV-1 and HIV-2 infections are prevalent. Although totally unexpected, our findings are also highly encouraging with regard to the promising vaccine vehicles used. ALVAC, in addition, has the advantage that no previous immunity to the vector has been established in the human population, in contrast to NYVAC, which is derived from the widely used vaccinia virus vaccine strain.

Our results must be interpreted with some caution, however. No statistical significance could be applied because of the small number of macaques studied. Moreover, the degree of protection observed was modest and the 6-month observation period short in view of recent observations in which some macaques inoculated intrarectally with low dose SIV remained apparently virus-free by the criteria of both virus isolation and PCR. Yet, they subsequently exhibited CD4 cell depletion and died of opportunistic infections (L. Kuller *et al.*, manuscript submitted). Thus, more stringent criteria for protection from virus infection may ultimately be required. The animal model used in these studies must also be considered in evaluating the results. HIV-2 does not give a robust infection in rhesus macaques, and therefore protection may have been more easily achieved. A strain of HIV-2 pathogenic for macaques may soon be available for future studies, providing a better model for HIV-1 in humans. In the absence of a perfect model, however, clues to vaccine protection must be gleaned from as many sources as possible for eventual application in human trials. Future studies using larger numbers of macaques and more in-depth immunological studies are planned. We also hope that this report stimulates investigators to perform similar studies in other heterologous systems.

Methodology

Immunogens. The NYVAC- and ALVAC-based HIV-1 recombinant viruses were generated by standard *in vitro* recombination assays[33] using NYVAC (vP8966) or ALVAC (CPpp) as rescue virus[8,9] and donor plasmids engineered for insertion of specific HIV-1 coding sequences. In all cases envelope expression cassettes were regulated by the vaccinia virus H6 promoter[34] and Gag expression cassettes were under the control of the vaccinia virus 13L promoter[35]. HIV-1_{IIIB} sequences were derived from plasmid pHXB.2 (ref. 36) while HIV-1_{MN} sequences were derived from plasmids pMN1.8-9 and pMN1.8-10. NYVAC-HIV-1 recombinant immunogens included two containing HIV-1_{IIIB} genes: vP911, containing the entire envelope gene for expression of gp160 (ref. 37); and vP921, encoding gp120 (amino acids 1 to 511). Four NYVAC-HIV-1 recombinants contained HIV-1_{MN} genes and were designed to express gp120 or gp160 in a secreted form or attached to the cell membrane: vP994, encoding a non-cleavable, truncated, secreted form of the envelope glycoprotein engineered by site-directed mutagenesis to change amino acid 511 from an arginine to a threonine[38] and eliminating the transmembrane anchor sequence (terminates at nucleotide 7808); vP1004, encoding gp120; vP1009, encoding the entire gp160 molecule in addition to the *gag/pol* genes; and vP1035, encoding the gp120 moiety fused to the hydrophobic transmembrane anchor sequence from the HIV-1 envelope glycoprotein (amino acids 711 to 728). The numbering of amino acids is according to Ratner *et al.*[36] Four HIV-1_{MN} recombinants were similarly constructed in the ALVAC vector: vCP120, equivalent to vP994 and encoding the truncated, secreted form of gp160; vCP124, equivalent to vP1004 and encoding gp120; vCP130, equivalent to vP1009 and encoding gp160 plus the *gag/pol* genes; and vCP138, equivalent to vP1035 and encoding gp120 plus the transmembrane anchor sequence.

Subunit immunogens included peptides and native or recombinant proteins. A mixed octameric V3 loop peptide preparation con-

Table 2 Summary of immune responses in vaccinated rhesus macaques

			Pre-challenge			At challenge		Post-challenge					
			Proliferation (S.I.)	CTL (% Lysis)	Neutralization (titre)	Neutralization (titre)		Proliferation (S.I.)		CTL (% lysis)		Neutralization (titre)	
Recombinant immunogen	Subunit boost	Macaque no.				HIV-1	HIV-2	HIV-1	2	1	2	1	2
NYVAC IIIB env													
gp160	octapeptide	214L	19	13	40	–	–	2	3	–	–	–	–
gp160	native gp120	215L	4	10	475	–	–	–	–	–	–	–	1300
gp120	octapeptide	218L	23	9	20	–	–	–	–	–	–	–	>7290
gp120	native gp120	219L	8	12	180	–	–	–	–	–	–	–	–
$ALVAC_{MN}$													
env, gag, pol	T-helper-V3 peptide	234L	4	22	20	10	–	–	–	–	–	15	260
env, gag, pol	gp160 & p24	237L	4	9	35	25	–	2	–	–	–	10	750
$NYVAC_{MN}$													
env, gag, pol	T-helper-V3-peptide	238L	3	10	520	55	–	13	–	–	10	95	>7290
env, gag, pol	gp160 & p24	239L	9	7	20	–	–	2	–	–	–	–	–

Peak immune responses attained by each of the immunized macaques before, at the time of, and after challenge are tabulated. The dash (-) signifies neutralizing antibody titre of <10; stimulation indices (S.I.) of <2; and cell lysis of <5%.

sisted of six different preparations of a heptalysyl core bearing eight copies of a particular V3 loop peptide sequence[39]. The HIV-1 V3 loop sequences represented included those of the IIIB, MN, RF, SC, WMJ2 and SF2 isolates. A tandem T-helper-V3 peptide was composed of a conserved T-helper epitope from HIV-1_{IIIB} p24[40] conjugated to the V3 loop peptide of the MN strain with the sequence GPKEPFRDYV-DRFYKRIHIGPGRAFYTTKN. Native HIV-1_{IIIB} gp120 was purified from the supernatant media of infected cells[41]. Native HIV-1_{IIIB} p24 was purified from infected cell extracts as previously described[42]. A gp160 chimeric protein consisted of gp120 of HIV-1_{MN} and gp41 of HIV-1_{LAI} purified from the supernatant media of BHK-21 cells infected with vaccinia virus encoding the chimera (M.-P. K., pers. commun.).

Immunization regimens. Eight juvenile rhesus macaques were inoculated twice intramuscularly (i.m.) at monthly intervals with NYVAC- or ALVAC-HIV-1 recombinants. The NYVAC-HIV-1_{IIIB} recombinants, vp911 and 921, were given at a dosage of 10^7 pfu to each of two macaques (214L, 215L and 218L, 219L, respectively). The NYVAC- or ALVAC-HIV-1_{MN} recombinants were given as a mixture of four recombinants (vP994, vP1004, vP1009 and vP1035; or vCP120, vCP124, vCP130 and vCP138, respectively) to each of two macaques (238L and 239L for the NYVAC mixture, 234 and 237 for the ALVAC mixture) for a total dosage of 4×10^7 pfu. Subsequent subunit boosts, as indicated in Figs 1–4, were all formulated in alum and given i.m. They consisted of either 120 μg of the octameric peptide, 100 μg of native gp120, 125 μg of the tandem T-helper-V3 peptide or 100 μg of p24 plus 100 μg of the chimeric gp160. Subsequent recombinant boosts at 10^7 pfu unless otherwise indicated in the figure legends were given i.m. The precise timing of booster inoculations is shown at the top of Fig. 1–4.

Immunologic assays. A standard ELISA technique was used to detect binding antibodies to HIV-1_{IIIB} gp120 in macaque sera diluted 1:25 (ref. 42). Positive sera were further titrated to obtain gp120 binding titres. Binding titres to the V3 loop peptides of the IIIB and MN isolates were determined by ELISA as described previously[27]. Following challenge, a standard ELISA was used to detect serum antibody reactivity against disrupted virions of HIV-1 and HIV-2. Neutralizing antibody titres were determined as previously described[43] using frozen titred stocks of HIV-1_{IIIB} and HIV-$2_{SBL6669}$, or fresh supernatant media from H9 cells productively infected with HIV-1_{MN}. H9 cells were used as targets for the IIIB and MN assays and CEM × 174 (ref. 44) cells as targets for the SBL6669 assays. Neutralizing titres are defined as the reciprocal of the serum dilution at which the percentage of virus infected cells is 60% of the control level, following normalization of the data to control infectivity levels. Values reported are the means of at least two determinations.

T-cell proliferative responses were monitored by culturing 3×10^5 PBMC in 200 μl of RPMI 1640 containing 10% fetal calf serum and penicillin/streptomycin (R10 media) in the presence of 1 μg HIV-1 proteins or synthetic peptides for 5 days at 37 °C. Following challenge, HIV-2 proteins and peptides were also used as stimuli. [^{3}H]Thymidine (1 μCi per well) was added and the microtitre plates were incubated an additional 24 hours. The cells were harvested onto filter mats using a Skatron cell harvester, and thymidine incorporation was determined using a β plate counter (Wallac). Results of triplicate determinations were expressed as the stimulation index (S.I.), defined as incorporation in the presence of test antigen relative to incorporation in the presence of control peptide or R10 media alone. Antigens used as stimuli in proliferative assays for animals immunized with IIIB recombinants included baculovirus-produced HIV-1_{IIIB} gp160, and synthetic peptides including the V3 loop (NNTRKSIRIQRGPGRAFVTIGKIGC), the shorter "p18" V3 loop peptide[45], and 3 peptides corresponding to known helper epitopes of HIV-1: T1, T2 and Th4.1 (refs 46,47). Proliferative assays using cells from macaques immunized with MN recombinants used the chimeric MN/LAI gp160 and the MN equivalents of the antigens listed above. In addition, a gag HGP30 epitope peptide[48] was used with the sequence VHQRIEIKDTKEALDKIEEEQNKSKKKA. A peptide containing a scrambled "p18" sequence of the MN isolate was used as control. Following challenge the panel of stimulating antigens was expanded to include HIV-2 proteins and peptides: β-propiolactone-inactivated HIV-2 (ref. 49), baculovirus-produced HIV-2 gp160, 3 peptides representative of the HIV-2 V3 loop region (KI-INKKPRQAG*C, ITLMSGRRFHSQKIINKKPRQAG*C, and RRPENKTVVPITLMSGRRFHSQKIINKKPRQAG*C where the asterisked G has been substituted for W present in the HIV-2 sequence), a peptide equivalent to the HGP30 region of the HIV-1 Gag protein (LHAEEKVKDTEEAKRIVGRHL), and the HIV-2 equivalents of the T1 and T2 helper epitopes (VTEQAVEDVWNLFETSIKP and NYVPCHIEQI-INTWHKVGKNVYL). The number of different stimuli used at any given time point depended on the total number of PBMC available. Only the peak S.I. achieved with any of the stimuli at any given time is plotted in Figs 1–4.

For CTL assay, macaque PBMC were used as effectors and infected, chromium-51-labelled herpesvirus papio transformed B cells from each macaque were used as targets. Effector PBMC (5×10^6) were stimulated *in vitro* for 5 days in 5 ml R10 media containing 0.2 μg ml^{-1} of one or more of the antigens used in proliferative assays and interleukin-2 (10 units ml^{-1}). On day 5 of the *in vitro* stimulation, target cells were infected with appropriate HIV-1 or HIV-2 recombinants at a multiplicity of infection of 10 for 1 hour at 37 °C. Infected cells were washed and labelled with ^{51}Cr (10 μCi per 10^6 cells per ml) overnight at 37 °C. For assessment of CTL activity in macaques immunized with NYVAC-HIV-1_{IIIB} recombinants, targets were infected with the ALVAC-based recombinant, vCP112 containing the IIIB envelope gene, in order to minimize detection of responses specific for the vector. For macaques immunized with either NYVAC- or ALVAC-HIV-1_{MN} recombinants, targets were infected with the ALVAC-HIV-1_{MN} recombinant, vCP130, expressing the *env* and *gag/pol* genes, or the equivalent NYVAC-HIV-1_{MN} recombinant, vP1009, respectively. Following challenge, targets were also prepared using vCP153 containing the HIV-2 *env* and *gag/pol* genes. The ALVAC (CPpp) and NYVAC (VP866) vectors without inserted genes served as controls. An alternative procedure to poxvirus-infected targets used target cells coated with either V3, "p18", or HGP30 peptides. Target cells were labelled overnight with ^{51}Cr and washed. Fifty microlitres of the cells (5×10^4/ml) were then pulsed with 50 μl of any of the synthetic peptides (20 μg ml^{-1})[50]. In either case, CTL activity was subsequently assessed by mixing effector and poxvirus-infected or pulsed target cells at various E:T ratios in duplicate and incubating for 4 to 6 hours at 37 °C. Control wells included targets with no effectors for determining spontaneous release, and target cells in the presence of 0.5% NP-40 for determining total release. Supernatant medium was harvested using a Skatron harvester, and ^{51}Cr released was counted in a gamma counter. Specific % lysis was calculated according to the formula: (experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm) × 100. Values are reported for E:T ratios of approximately 100:1, and represent the highest specific per cent lysis obtained at each time point with any viral-specific target cell. In Figs 1–4, per cent lysis obtained with control targets has been subtracted. Values greater than 10% net specific lysis are considered positive.

HIV-2 challenge. Five months following the last boost with HIV-1 recombinant, the eight immunized macaques and three naive controls (J198, J190 and L1) were challenged intravenously with HIV-$2_{SBL6669}$

grown in macaque PBMC. The viral stock, previously titred in cynomolgus macaques, was first retitred in six naive rhesus macaques. Two macaques infected with 250 cynomolgus monkey infectious doses (MID) and two infected with 125 MID quickly became infected; two macaques infected with 62.5 MID also became infected, one with a delay. Therefore, 100 MID was selected as the challenge dose.

Isolation of HIV-2 was carried out on unfractionated macaque PBMC as well as $CD8^+$-depleted PBMC at the times indicated in Table 1. Lymphocytes activated with 2 μg ml^{-1} phytohaemagglutinin for 48 hours were co-cultured with similarly activated polybrene-treated human PBMC. Fresh target cells were added weekly, and virus expression was monitored by p24 antigen capture.

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