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Evaluation of recombinant Kunjin replicon SIV vaccines for protective efficacy in macaques

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Abstract

Persistent gag-specific T cell immunity would be a useful component of an effective HIV vaccine. The Flavivirus Kunjin replicon was previously engineered to persistently express HIV gag and was shown to induce protective responses in mice. We evaluated Kunjin replicon virus-like-particles expressing SIVgag–pol in pigtail macaques. Kunjin-specific antibodies were induced, but no SIV-specific T cell immunity were detected. Following SIV_{mac251} challenge, there was no difference in SIV viremia or retention of CD4 T cells between Kunjin–SIVgag–pol vaccine immunized animals and controls. An amnestic SIV gag-specific CD8 T cell response associated with control of viremia was observed in 1 of 6 immunized animals. Refinements of this vector system and optimization of the immunization doses, routes, and schedules are required prior to clinical trials.

Keywords: Kunjin; HIV; SIV; Macaques; *Macaca nemestrina*; Vaccines

Introduction

HIV infections remain a major threat to global health. A prophylactic vaccine is urgently required, but attempts to induce broadly reactive neutralizing antibodies or protective T cell immunity have thus far failed (2007; Cohen, 2003). Most studies have shown that following HIV-1 infection of humans and SIV infection of macaques, CD8 and CD4 T cell responses correlate with control of acute infection, lowered viremia and in some situations improved survival (Johnston and Fauci, 2007; Schmitz et al., 1999). Several studies have shown that T cell immunity to SIV or chimeric SHIV induced by vaccination does not prevent infection but results in reduced levels of viremia and prevention of immunodeficiency in macaques (Amara et al., 2001; Barouch et al., 2000; Dale et al., 2004; Wilson et al., 2006).

There has been recent interest in several qualitative aspects of HIV or SIV-specific CD4 and CD8 T cell immunity. Gag-specific T cell immunity may be more beneficial than T cell responses to other antigens (Kiepiela et al., 2007; Sacha et al., 2007). Expression of multiple effector cytokines, chemokines and degranulation molecules from HIV-specific T cells has also been shown to be beneficial (Betts et al., 2006). Generation of long-lived T cells with the appropriate effector and memory phenotypes may be required for durable immunity (Almeida et al., 2007; Fernandez et al., 2007; Sun et al., 2005).

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Many viral and bacterial vectors have been utilized to express HIV or SIV antigens from within cells and thereby prime T cell immunity. Replication-incompetent adenovirus- and canarypox-HIV vaccine vectors have entered advanced clinical trials (Johnston and Fauci, 2007). Most recombinant vector-based vaccine studies have not shown as efficient in control of virulent SIV challenge as live attenuated SIV vaccines for reasons that remain unclear (Koff et al., 2006). We recently showed that recombinant poxvirus vector-based vaccines, which typically express the inserted SIV genes only transiently, induce SIV-specific CTLs with slow kinetics of killing in comparison to live attenuated vaccines (Rollman et al., 2007). There has been recent interest in replication-competent virus vectors that can persistently express the inserted HIV/SIV antigens (Evans et al., 2005; Kaur et al., 2007). Persistent vectors however face tough regulatory hurdles for clinical trials. Safer vectors incapable of *in vivo* packaging and spread that express high levels of persisting antigen are needed.

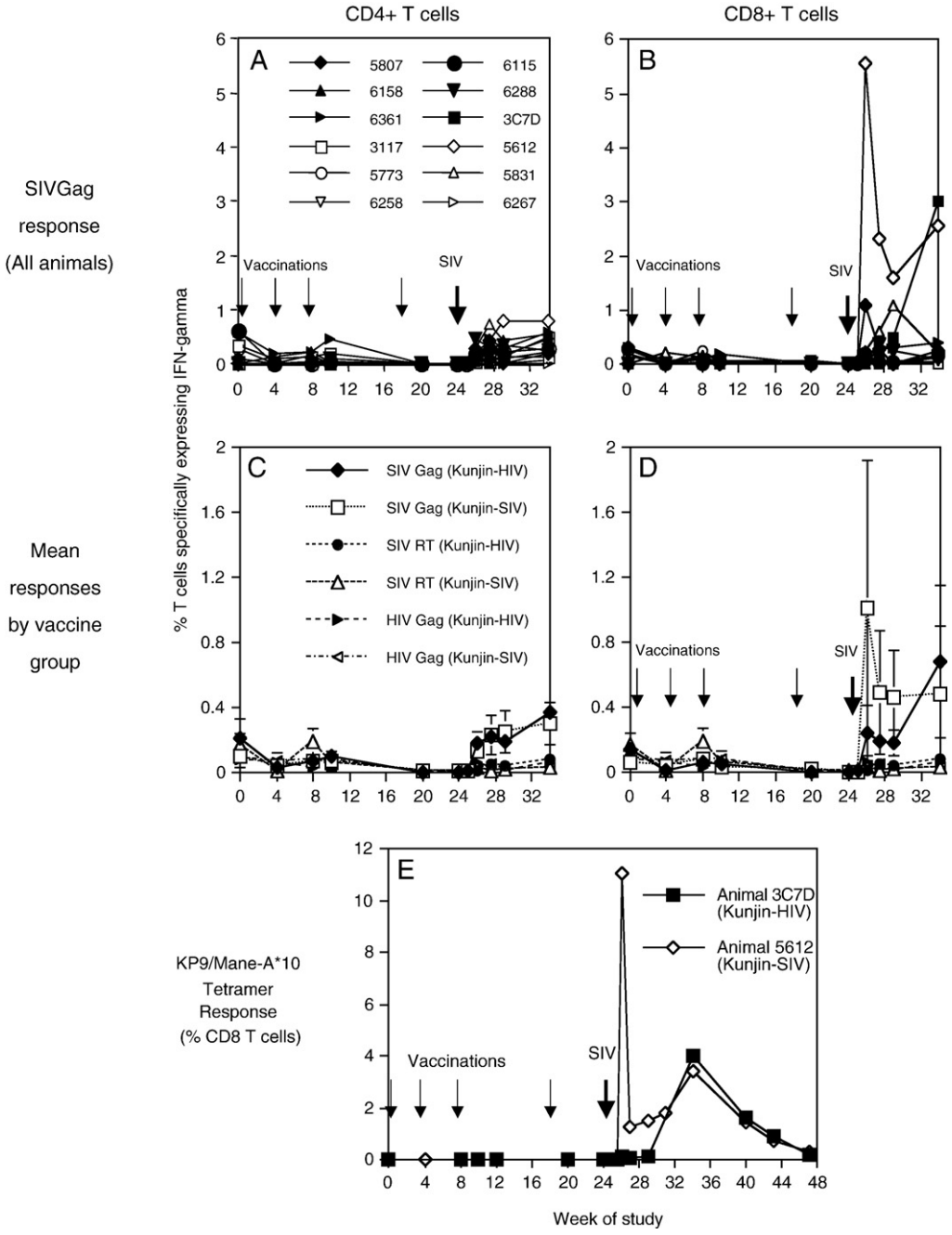

Fig. 1. T cell immunogenicity of Kunjin–SIV/HIV vaccines. 12 animals were vaccinated (small arrows) with Kunjin–SIV gag/RT vaccines (open symbols) or Kunjin–HIV gag vaccines (closed symbols). Antigen-specific IFNγ expression from CD4+3+ or CD8+3+ T lymphocytes was assessed on fresh blood by intracellular cytokine staining. All animals were challenged with SIV_{mac251} at week 24 (large arrow) A. SIV gag-specific CD4 and B. CD8 T cell responses from individual animals. Kunjin–SIV-vaccinated macaques have solid symbols and Kunjin–HIV-vaccinated macaques have open symbols. C. Mean SIV gag-, SIV RT- and HIV-1 gag-specific CD4 and D. CD8 T cell responses by vaccine group. E. CD8 T cells to the SIV gag epitope KP9 were tracked in 2 *Mane-A*10+* animals (one in each vaccine group) by a Mane-A*10/KP9 tetramer.

Alphavirus replicon vectors have attracted interest as HIV vaccines since they can be engineered to express high levels of antigens. Venezuelan Equine Encephalitis, Sindbis and other alphavirus replicon vectors have shown induction of some T cell immunity in macaques (Davis et al., 2000; Gupta et al., 2006; Xu et al., 2006). A potential disadvantage of alphavirus replicon vectors is the transient antigen expression following apoptosis of infected antigen-expressing cells and high frequency of recombination during VLP preparations giving rise to infectious recombinant viruses. Replicons of an Australian Flavivirus Kunjin has a diminished capacity to induce apoptosis in infected cells, thereby allowing prolonged antigen expression and induction of high levels of T cell immunity (Anraku et al., 2002; Harvey et al., 2003; Herd et al., 2004). Kunjin–HIVgag vaccine vectors have induced high levels of long-lived CD8 T cell immunity in mice (Harvey et al., 2003). The promise of the current-generation of Kunjin replicon–HIV vaccine vectors thus warranted evaluation in primates.

Results and discussion

We randomized twelve pigtail macaques 1:1 to receive either Kunjin VLPs expressing either SIV gag/RT or HIV gag (control group) 4 times at 0, 4, 8 and 18 weeks (see arrows, Fig. 1A). The vaccinations were safe, not inducing marked injection site reactions or changes in hematology profiles (not shown).

Following the initial and the 3 booster vaccinations, no significant HIV or SIV-specific CD4 or CD8 T cell responses were detected by IFNγ intracellular cytokine analyses on fresh whole blood (Fig. 1). In addition, no responses were found by staining for the cytokines TNFα or IL-2 (not shown). No SIV gag KP9-specific responses were detected in the 2 *Mane-A*10+* animals by KP9/Mane-A*10 tetramer staining prior to SIV challenge (Fig. 1E).

To study humoral immunity following vaccination with KUN-SIVgag/pol vaccine, we examined SIV-specific antibodies following the vaccinations by Western Blot as previously described (Batten et al., 2006) (Fig. 2). No SIV gag-specific antibodies appeared to be induced by vaccination — some non-specific p24/p26 gag bands were present at baseline that did not expand after vaccination. Two weeks following the 3rd and 4th vaccinations (week 10 and 20, lanes 1, 2 in Fig. 2), bands were present to the p66/68 region in both Kunjin–SIV and Kunjin–HIV vaccinated macaques. These bands were not expanded after SIV challenge (lanes 4–6, Fig. 2). Similar bands were detected after vaccination in Kunjin–HIV vaccinated using a HIV-1 Western Blot (not

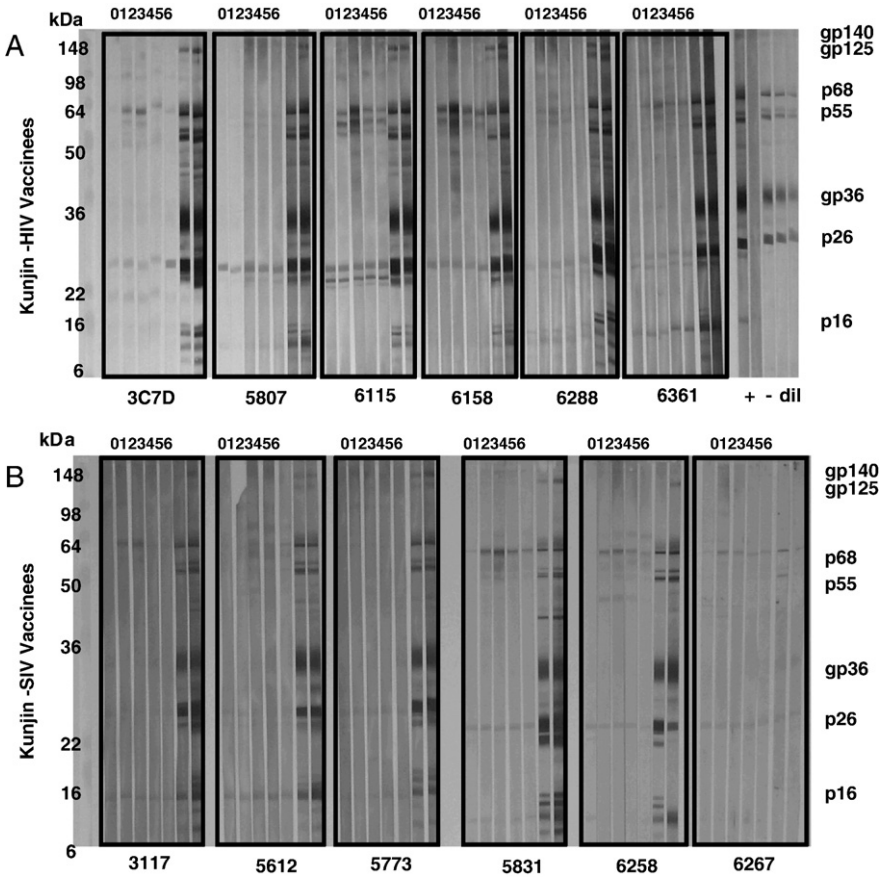

Fig. 2. Humoral immunity of Kunjin–SIV vaccine. Western Blots (WB) for antibodies to SIV antigens were performed from plasma from pre-vaccination (lane 0, week 0), 2 weeks after the 3rd vaccination (lane 1, week 10), 2 weeks after the 4th vaccination (lane 2, week 20), on the day of SIV_{mac251} challenge (lane 3, week 24), 2 weeks after challenge (lane 4, week 26), 7 weeks after challenge (lane 5, week 31) and 16 weeks after challenge (lane 6, week 40). HIV-2 Western Blots to detect SIV-specific antibodies are shown on Kunjin/HIV-vaccinated (A) and Kunjin/SIV-vaccinated (B) macaques. Size standard are shown at the left and positive (+) and negative (−) control human sera, together with a serial 2-fold dilution (dil) of the positive sera and shown at the right of panel A.

shown). The Kunjin–HIV vaccine does not express Pol antigens suggesting the bands were not specific to Pol antigens and may represent cross-reactive responses to Kunjin virus antigens.

Although no T and B cell immune response to SIV antigen were detected after the Kunjin VLP vaccinations, it was possible that some low-level responses were primed. To address this possibility the macaques were tested for vector-specific immune responses using an overlapping 18 mer peptide library covering the Kunjin virus NS3 protein. NS3 is expressed intracellularly by the replicon and known to be a target of Flavivirus-specific T cell immunity in mice and in humans (Appanna et al., 2007; Hill et al., 1992; Kumar et al., 2004; Simmons et al., 2005). Kunjin NS3-specific T cell immunity was examined by ICS at multiple time points during the course of vaccination. No significant CD4 or CD8 T cell immune responses were detected to Kunjin NS3 peptides in any of the 12 Kunjin–HIV and Kunjin–SIV immunized animals (<0.1% of CD4 or CD8 T cells expressing IFNγ). To assess humoral immunity elicited to the inoculated VLPs, we studied neutralizing antibodies to Kunjin virus directed against envelope protein present as structural component of VLPs but not expressed by the replicon (Hall et al., 2003). As expected, Kunjin-specific neutralizing antibodies were detected after vaccination in all 12 animals after 3 vaccinations, with no overall differences between the 2 vaccine groups (Fig. 3). There were modestly higher Kunjin-specific antibodies detected in the Kunjin–HIV vaccines after 1 vaccination (week 4, Fig. 3B), potentially reflecting the higher dose of VLPs used in the Kunjin–HIV vaccinations. After 3 vaccinations the Kunjin antibody responses were similar between the 2 vaccine groups suggesting an adequate dose of VLP vaccination was used for both vaccine groups.

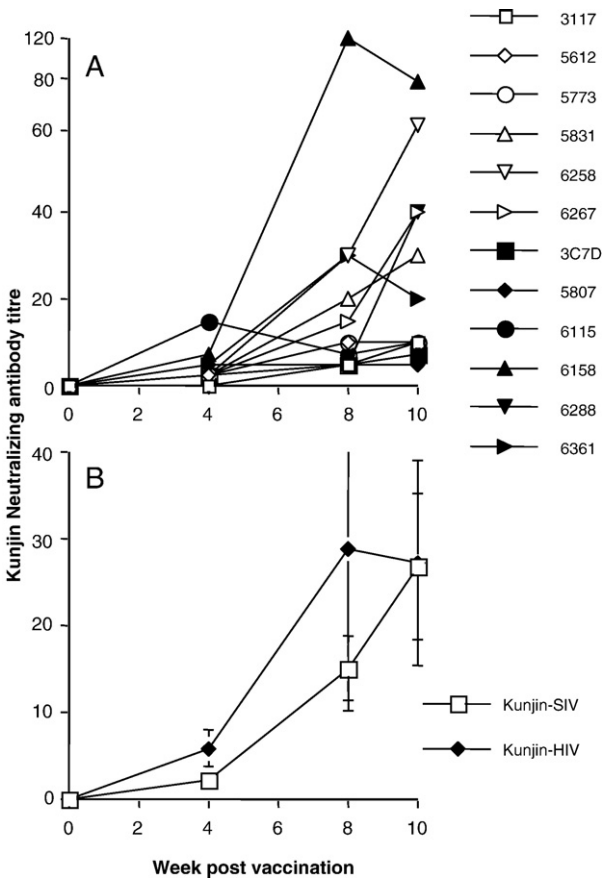

Fig. 3. Kunjin virus specific neutralizing antibodies. Neutralizing antibodies were followed after the 3rd vaccination. A. All animals are shown — Kunjin–SIV-vaccinated macaques have open symbols and Kunjin–HIV-vaccinated macaques have closed symbols. B. Mean (±SE) of both vaccine groups is shown.

At week 24 after the initial vaccination, all 12 animals were challenged intravenously with a pathogenic stock of SIV_{mac251} (Batten et al., 2006). All macaques became infected, with mean peak viral loads 2 weeks after inoculation in all 12 animals of 7.0 (range 6.1 – 8.1) $\log_{10}$ copies of SIV RNA/ml plasma. There was no significant difference in mean viral loads between Kunjin–SIV-vaccinated animals and Kunjin–HIV-vaccinated animals out to 23 weeks after infection (Fig. 4A). Mean peak and set point (between weeks 5–23 after infection) viral loads between vaccinated and control animals were almost identical (7.1 vs. 6.9 and 5.6 vs. 5.5 $\log_{10}$ copies/ml respectively). Only one macaque (the *Mane-A*10*+ animal in the Kunjin–SIV vaccinated group, animal 5612) subsequently controlled its viral load (Fig. 4B). A gradual decline in peripheral CD4 T cells was observed in both groups, without significant differences between the groups (Figs. 4C, D). Four of the 12 macaques (2 in each vaccine group) were euthanised prior to 23 weeks of follow-up. All 4 euthanised macaques had high viral loads (>6.0 $\log_{10}$ copies/ml), CD4 T cell depletion (<20%), failure to gain weight and thrombocytopenia (<100/μl), consistent with incipient AIDS.

To further address whether SIV-specific immune responses were primed by vaccination, we analyzed whether a sharp anamnestic T cell and antibody response was observed early after challenge. No overall differences in SIV-specific CD4 or CD8 T cell responses after challenge were observed between the 2 vaccine groups (Figs. 1A–D). No rapid recall response of SIV gag antibodies was observed in the SIV vaccine group (Fig. 2). One Kunjin–SIV vaccinated *Mane-A*10*+ macaque (animal 5612) did however have a large (>10%) SIV gag KP9 epitope-specific response detected early (within 2 weeks) after challenge (Fig. 1E). The KP9 response was likely primed by vaccination since (a) the control *Mane-A*10*+ animal that received the Kunjin–HIV vaccine had no early rise in KP9-specific CD8 T cells and (b) our previous experience with 24 *Mane-A*10*+ macaques demonstrated a slow pattern of generating KP9-specific CD8 T cells in the absence of prior vaccination (Fernandez et al., 2007). One Kunjin–HIV vaccinated animal (#5807) generated a 1.0% SIV gag-specific CD8 T cell response early after challenge (black diamonds, Fig. 1B), but this was not sustained and likely reflects a *de novo* response to SIV infection.

In summary, although Kunjin replicon VLP vaccines expressed HIV and SIV gag efficiently *in vitro* and are immunogenic in mice, this was a largely negative macaque study. With the exception of the *Mane-A*10*+ animal (see below), no induction of SIV-specific immunity was detected in pigtail macaques and no protection from a pathogenic SIV_{mac251} challenge was observed. No Kunjin vector-specific T cell responses were detected prior to challenge, although neutralizing antibodies to the structural component of the Kunjin replicon delivery vehicle (VLPs) were readily detected in all animals. This is consistent with extracellular recognition of the

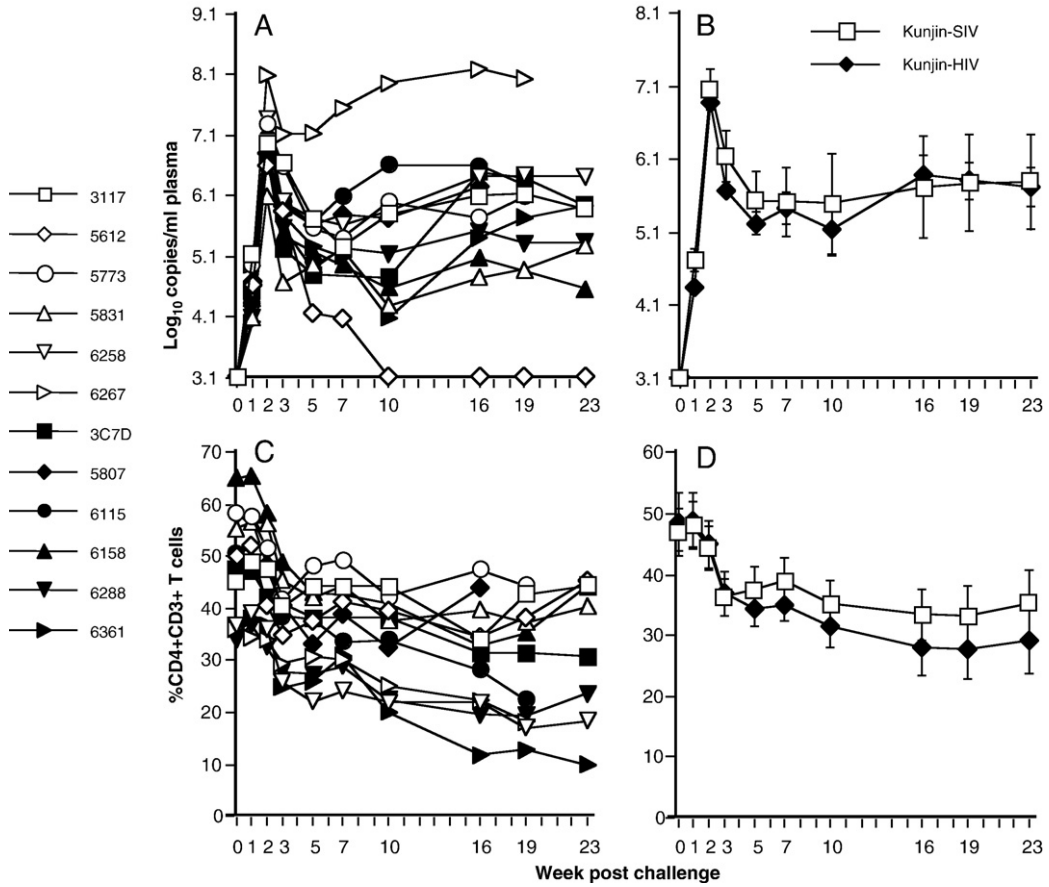

Fig. 4. Outcome of SIV_{mac251} challenge. Plasma viremia and peripheral CD4 T cell levels followed after challenge for 23 weeks. A, C. All animals are shown — Kunjin–SIV vaccinated macaques have open symbols and Kunjin–HIV vaccinated macaques have closed symbols. B, D. Mean (±SE) of both vaccine groups is shown. 4 animals (2 in each group) were euthanised prior to the completion of the study and the last-observation is carried forward for the mean of the groups for the last 2 time points.

inoculated VLPs but limited intracellular expression of either Kunjin or SIV genes. Apparent blockade of entry or replication of the Kunjin replicons in primates under immunization conditions used in this study was not expected and is currently under further evaluation. Mouse experiments suggest that efficient vaccination is achieved with intraperitoneal administration, which may have elicited better responses in macaques compared to the intramuscular or subcutaneous administrations used in this study. Antigen-presenting cells of specific types or local concentrations of antiviral responses at the sites of injection may be important mediators of vaccine efficacy of this (and perhaps other) replicon system.

No early anamnestic T cell or antibody responses to the SIV_{mac251} challenge were observed in 5 of the 6 Kunjin–SIV vaccinated animals. However, the one *Mane-A*10*+ Kunjin–SIV vaccinated animal (#5612) had a rapid rise in SIV gag-specific CD8 T cells early after challenge, detected by both ICS (5.5% of CD8 T cells expressing IFNγ to gag) and Mane-A*10/KP9 tetramer (11% of CD8 T cells). This is consistent with priming of SIV gag KP9-specific CD8 T cells by vaccination, although we did not detect priming with our whole blood ICS or tetramer assays. We also performed a cultured IFNγ ELISpot in an attempt to detect low-level primed responses by adapting a published human assay (Goonetilleke et al., 2006). Again we did not detect any SIV-specific responses in the 12 animals, although the assay could require further optimization and validation for macaque cells (not shown). Nonetheless, Kunjin–SIV vaccinated animal 5612 subsequently controlled SIV_{mac251} viremia within 10 weeks following challenge and did not experience a CD4 T cell decline. Although this single animal suggests that effective priming of SIV-specific immunity may be possible with Kunjin–SIV vaccines, improvements and further optimization of vector preparations, immunization doses, routes and schedules are clearly needed to more broadly and robustly induce immunity to SIV antigens in primates with this technology.

Materials and methods

Vaccines

Kunjin virus replicon vaccines, KUN–SIVgag–pol, expressing SIV_{mac239} gag matrix and nucleocapsid connected in frame with reverse transcriptase (RT) and KUN–HIV gag expressing HIV-1 gag were constructed as described elsewhere ((Harvey et al., 2003), Anraku et al. submitted). The Kunjin replicon vaccines were produced in the form of virus-like particles (VLPs) by transfecting KUN–SIVgag–pol replicon RNA into tet-KUNCprME packaging cells as described previously (Harvey et al., 2004). The VLPs were concentrated by tangential flow

filtration (300 kDa cut off) and then spinning the preparation through a sucrose cushion (12.5% sucrose and 2.5% trehalose). The pellets were resuspended in DMEM and frozen at− 70 °C in aliquots. Antigen expression from both vaccines *in vitro* was confirmed as previously described ((Harvey et al., 2003), Anraku et al. submitted).

Macaques

Twelve juvenile pigtail macaques (*Macaca nemestrina*) were studied in a protocol approved by the University of Melbourne and CSIRO Livestock Industries animal ethics committees. Macaques were stratified by weight, gender and the MHC class I allele *Mane-A*10* (1 animal each group, 5612 in the Kunjin–SIV group and 3C7D in the Kunjin–HIV group) identified as previously described (Smith et al., 2005b). Mane-A*10 presents the immunodominant KP9 SIV gag epitope and results in delayed progression to AIDS (Smith et al., 2005a). Immunizations were given in equal doses (1.2×10^8 VLPs for Kunjin–SIV and 8×10^8 VLPs for Kunjin–HIV in 1 ml) by the intramuscular and subcutaneous routes over the thigh in opposite legs.

Immunogenicity testing

SIV- and HIV-specific T cell responses were assessed by intracellular cytokine staining for IFNγ expression. Fresh whole blood (200 μl) was stimulated for 6 h with overlapping 15 mer peptide sets spanning SIV_{mac239} gag, SIV_{mac239} RT, or HIV-1 consensus subtype B gag (all from NIH AIDS Reagent repository) as described (De Rose et al., 2007). We also examined fresh blood for SIV gag KP9-specific CD8 T cells by staining with the Mane-A*10/KP9 MHC tetramer in the 2 *Mane-A*10*+ animals as previously described (Fernandez et al., 2007). SIV-specific antibodies were assessed in macaque sera samples by Western Blot to a HIV-2 antigen preparation as described (Batten et al., 2006). Macaque blood was also tested for vector-specific immune responses by intracellular cytokine staining for IFNγ expression using an overlapping 18 mer peptide library covering the Kunjin virus NS3 protein (88 18 mer peptides overlapping by 11) (GL Biochem, Shanghai, China). Serum neutralizing antibodies to Kunjin virus directed against envelope protein present as structural component of VLPs were assessed as previously described (Hall et al., 2003).

Virus challenge

At week 24 after the initial vaccination, all 12 animals were challenged intravenously with a pathogenic stock of SIV_{mac251} (40 TCID50) we have used previously in pigtail macaques (Batten et al., 2006). We monitored SIV RNA in plasma and peripheral CD4 T cell depletion as previously described (De Rose et al., 2007).

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