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Kunjin replicon-based simian immunodeficiency virus gag vaccines

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A number of RNA-based vector systems have been used in the construction of HIV-1 and SIV vaccines. These include vectors based on negative strand RNA viruses, positive strand RNA viruses, and double-stranded RNA replication intermediates. We have previously described a replicon vector system based on the Kunjin flavivirus, which encodes SIV gag-pol using an RNA-optimised nucleic acid sequence. The resulting VLP vaccines are capable of inducing potent, long-lived and protective CD8 T cell responses in several murine systems. Kunjin replicon VLP vaccines encoding HIV-1 gag have also been shown to induce CD8 T cell immunity comparable to that seen after immunisation with recombinant vaccinia. In contrast to most alphavirus replicon-infected cells, Kunjin replicon-infected cells do not show overt cytopathic effects, and both double strand RNA-induced “danger signals” and antigen production are maintained for extended periods. Herein we describe the construction and immunogenicity of four SIV gag-pol Kunjin replicon VLP vaccines. The first encodes SIV gag using the wild-type nucleic acid sequence (WT). The second encodes SIV gag using an RNA-optimised nucleic acid sequence (DX). HIV and SIV gag contain RNA inhibitory sequence (INS) elements, which have been shown to act at several levels to inhibit gag expression; primarily via nuclear retention of RNA in the absence of the Rev protein. RNA-optimisation strategies, which remove these INS elements by changing the codon usage, result in significant increases in protein expression from DNA-based expression vec-
tors [22], and such RNA-optimised SIV gag vaccines have been used in a number of monkey trials [23,24]. Inhibition of nuclear export of cytotoxic lymphocyte Kunjin replicon RNA, however, INS elements have also been reported to reduce RNA stability and translation [25,26]. The third SIV gag Kunjin replicon VLP vaccine encodes a human codon-optimised SIV gag gene (OPT). Codon optimisation has been used extensively to improve recombinant antigen expression in a number of systems and the process involves altering the codon usage to the one most commonly utilized in a given species [27,28]. The fourth vaccine encodes wild-type matrix and capsid from gag-linked in-frame to reverse transcriptase from pol (Gag-pol). This approach has been described previously for HIV Gag and removes from the gag gene sequences that encode proteins with potentially immunosuppressive properties, whilst retaining sequences frequently recognised by T cells [29]. Although all four vaccines expressed Gag protein at comparable levels, they behaved quite differently in terms of immunogenicity, protection and insert stability.

2. Materials and methods

2.1. Plasmids and construction of Kunjin SIV gag (pol) vaccines

The RNA-based Kunjin replicon vector C20ubHDVrrep (SP6KUNrep1) [30] was used to construct WT, DX and OPT vaccines, and SP6KUNrep6 [15] was used to construct the Gag-pol vaccine. These two RNA-based Kunjin replicon vectors are the same, except SP6KUNrep6 has two copies of the foot and mouse virus 2A autoprotease, one upstream of MluI (replacing the ubiquitin gene) and another one downstream of the SnaBl cloning sites [12]. The Kunjin SIV gag WT construct encodes SIV mac239 gag subcloned from p239SpSp5 [31] (catalogue #829, NIH AIDS Research & Reference Reagent Program, Germantown, MD, USA) using Ascl sites. The Kunjin SIV gag DX construct was generated by subcloning RNA-optimised SIV mac239 gag DX [23,24] from pFB-SIVgag DX CTE Neo [32] using Ascl/SnaBl sites. Kunjin SIV gag OPT was constructed using Ascl/SnaBl sites and p01-426 (catalogue #9422, NIH AIDS Research & Reference Reagent Program), which encodes human codon-optimised SIVmac239 gag. The Kunjin SIVmac239 Gag-pol vaccine was constructed by separately PCR-amplifying matrix (MA)/nucleocapsid (NC) and reverse transcriptase (RT) genes from p239SpSp5 plasmid (NIH AIDS Research & Reference Reagent Program) and joining at SacII. The fragment was then cloned into the Mlu I and SnaB I sites of SP6KUNrep6 vector. Construction of the HIV-1 gag vaccine has been described previously and was expressed from the SP6KUNrep1 vector [11]. All gag constructs used in this study-encoded alanine in place of the initial glycine in Gag.

2.2. Production of virus-like particles (VLPs) and determination of their titre

Kunjin replicon RNAs were transcribed in vitro using SP6 RNA polymerase and electroporated into tettKUNCpmR BHK packaging cells as described previously [14]. Briefly, RNA (20–30 μg) was electroporated into 3×10^7 packaging cells in suspension and the cells were seeded into a 100-mm dish and incubated at 37 °C for 3–5 days and culture fluid collected at days 3–5. The titre of infectious VLPs in infectious units (IU) was determined by infection of Vero cells with 10-fold serial dilutions of the culture fluid. At 30–40 post-infection the cells were fixed with methanol/acetone (1:1, v/v) and analysed by immunofluorescence antibody staining using the 4G4 monoclonal anti-NS1 antibody [33,34] and the 55-2F12 anti-SIV Gag monoclonal antibody (catalogue #1610, NIH AIDS Research & Reference Reagent Program) and the number of positive cells counted using Zeiss Axioscop 2 fluorescent microscope (Carl Zeiss Microimaging GmbH, Berlin, Germany).

2.3. Detection of SIV gag and Kunjin NS5 protein by western blot

Vero or BHK cells were infected with VLPs at MOI = 1 or electroporated with 5–10 μg of Kunjin SIV gag RNA constructs and cultured for 2 days in a 60-mm dish. The cells were lysed with RIPA buffer and cell debris was removed by centrifugation. The protein concentration for each sample was determined using the BioRad Protein assay (Biorad, Regents Park, NSW, Australia). Fifteen microgram of total cell protein was separated on a 12.5% gel by SDS-PAGE and transferred onto Hybond-P membrane (Amer sham-Pharmacia Biotech, UK) using the Mini Trans Blot transfer tanks (Biorad, Regents Park, NSW, Australia) at 4 °C.

The His-tagged recombinant SIV mac239 and HIV-1 (clade B SF2/BH10) gag proteins were expressed from the pET28a+ plasmid (Merck Pty. Ltd., VIC, Australia) under control of T7-promoter in Escherichia coli C43(DE3) [35] and purified using a Ni-agarose column. Gag proteins were detected using the 55-2F12 or the AG3.0 monoclonal antibody (catalogue # 4121, NIH AIDS Research & Reference Reagent Program). Kunjin NS5 protein was detected by an anti-Kunjin NS5 monoclonal antibody (SH1.1) (Hall et al., in press). The secondary antibody was sheep anti-mouse-HRP (Chemicon, Melbourne, Australia). The proteins were visualised by the Western Lightning Chemiluminescence Reagent (PerkinElmer, Boston, MA, USA).

2.4. Deletion mutant analyses

Vero cells were transfected with Kunjin SIV gag VLPs at MOI = 1 and were cultured for 72 h. Total RNA was collected using Trizol reagent (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen) was used to produce cDNA. PCR primers that bind to C20 and E22 of the Kunjin replicon vector backbone were then used to amplify fragments from the cDNA using DyNAzyme II Taq (Finnzymes, Diagnostics, Espoo, Finland); forward primer 5’ gcgtgt- caaatgctaaacg 3’, reverse primer 5’ tcttcttctcgtgaagct 3’.

2.5. Vaccination of mice

Female BALB/c (H-2d) mice (6–8 weeks) were supplied by the Animal Resources Centre (Perth, Western Australia). Mice were vaccinated with the Kunjin replicon SIV gag VLP vaccines WT, OPT, DX, or Gag-pol, the Kunjin HIV-1 gag VLP vaccine [11] or control VLPs diluted in RPMI 1640 and 10^6 IU injected by the intraperitoneal (i.p.) route in a final volume of 200 μl.

2.6. ELISPOT assays

Interferon γ (IFNγ) ELISPOT assays were conducted using 125 or 123 overlapping 15-mer peptides spanning the entire SIVmac239 or HIV-1 subtype B gag protein, respectively (catalogue #6204 and 8117, respectively, NIH AIDS Research & Reference Reagent Program). The peptides were divided into six pools and the total final concentration of the peptide mix in each pool was 10 μg/ml. For Kunjin-specific responses directed to NS3, GYISTRVEL (H-2Kd) was used (AusPep, Parkville Victoria, Australia). Ex vivo ELISPOT assays were conducted as described previously [36] except that MultiScreen-IP hydrophobic PVDF membrane microtitre plates (Millipore Australia Ltd., North Ryde, Australia) and 25 IU/ml of IL-2 (kindly provided by Cetus Corp., Emeryville, CA, USA) was used.
The highest cell concentration was 2.5 \times 10^5 cells/well in triplicate, followed by four doubling dilutions. Spots were counted using a KS ELISPOT reader (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). Mean background spots from wells without peptide were subtracted from the mean number of spots obtained from wells with peptide.

Cultured ELISPOT assays were performed using splenocytes cultured in vitro for 6 days in 24 well plates (5 \times 10^6 cells/well in 1 ml medium) with a single pool of the entire overlapping gag peptides (10 \mu g/ml of the peptide pool) or GYSTRVEL (10 \mu g/ml). (Culture of splenocytes in the presence of 100 \mu g/ml of the SIV peptide pool resulted in excessive responses being detected in naive mice--data not shown). On day 4 a further 1 ml of medium was added to each well. On day 6 the cells were used in the ELISPOT assay (as described above) starting at 1.25 \times 10^5 cells per well followed by four doubling dilutions.

Groups (n = 3 or 4) were compared (using the total peptide response for each mouse) with the non-parametric Median test and the Monte Carlo significance given (SPSS for Windows, Version 15.0, 2007, SPSS Inc., Chicago, IL, USA).

2.7. Challenge assay using A20 cells expressing SIV gag

A plasmid encoding an EGF-SIV gag DX fusion protein was generated by subcloning human RNA-optimised SIV gag DX from the plasmid pCMVSIgagDX [24] (provided by Dr B. Felber) into Xhol/EcoRI sites of the pEGFP C3 plasmid (catalogue #6082-1. BD Biosciences, Franklin Lakes, NJ, USA) to generate pEGFP SIgagDX. This plasmid was used to transfect the BALB/c-derived A20 murine lymphoblastic leukemia cells (ATCC, TIB-208) using the GeneJammer Transfection Reagent (StrataGene, La Jolla, CA, USA). Cells were cultured for 24 h and sorted using MoFlo High-Performance Cell Sorter (Dako, Glostrup, Denmark) for high EGFP expression. The sorted cells were cultured in medium [37] containing G418 (800 \mu g/ml) for 1–2 weeks then sorted again for high EGFP expression. This procedure was repeated eight times at which point expression had stabilised with >95% of cells expressing EGFP as assessed by FACS after a week of culture under G418 selection. Expression of EGFP-SIV Gag DX fusion protein was also confirmed by western blot using anti-SIV Gag (55-2F12) and anti-EGFP antibodies (Molecular Probes, Leiden, Netherlands). (We were unable to generate a Gag-expressing line using J774 macrophage cells.)

Groups of BALB/c mice were vaccinated twice separated by 2–3 weeks with 10^6 IU of each of the four VLP vaccines. IU represents the titre of VLPs expressing Gag (see Table 2), so each mouse received the same number of Kunjin VLPs encoding gag. Two negative control groups were included to account for non-specific responses, one group received no vaccination (control naive) and the second was vaccinated with VLPs not encoding gag (control VLP). Immunisations with Kunjin VLPs encoding HIV-1 gag was also included. Ten weeks after the last immunisation splenocytes were assayed by ex vivo and cultured IFN\gamma ELISPOT assays using six pools of overlapping peptides spanning the entire SIV Gag protein or in the case of HIV gag immunised mice, the HIV Gag protein. The use of peptide pools covering Gag to measure T cell responses in mice has been described previously [38,39].

Ex vivo ELISPOT assays measure immediate effector function and thus tend to measure effector memory T cell activity, whereas cultured ELISPOT assays measure the ability of cells to replicate and form effector cells and thus represents a measure of central memory cell activity [40–42]. Ex vivo ELISPOT assays illustrated that responses induced by the WT vaccine were not significantly higher than the control VLP (p = 0.092) (Fig. 3A). DX induced responses that approached significance over control VLPs (p = 0.054), and OPT, Gag-pol and the HIV gag vaccine induced responses that were significantly higher than the control VLP (p = 0.047, 0.029 and 0.039, respectively) (Fig. 3A). The OPT and Gag-pol vaccine-induced responses were comparable to those induced by the Kunjin HIV-1 gag VLP vaccine tested under the same conditions (Fig. 3A). Using the cultured ELISPOT assay, WT and OPT failed to produce significant responses over the controls (Fig. 3B). The Gag-pol con-

with reverse transcriptase from pol. An HIV-1 gag construct encod-
struct induced significantly higher responses than WT (p = 0.015), whereas DX (due to the very high mouse to mouse variation in this group, ranging from 2688 to 17,000 spots) did not reach significance over WT using the Median test (although it did reach asymptotic significance using the Mann–Whitney test, p = 0.034). DX and Gag-pol vaccines also induced responses comparable to those induced by HIV-1 gag VLP vaccination (Fig. 3B). Pool 3 dominated the HIV-1 Gag responses (Fig. 3B) as this pool contains the immunodominant AMQMLKETI epitope [11].

3.3. Vector-specific CD8 T cell induction by Kunjin replicon VLP vaccines

Spaulding et al. identified a region in Dengue virus NS3, which represents a dominant CD8 T cell epitope [43]. We have confirmed that the homologous region (E205-GYISTRVEL-207) in Kunjin virus NS3, which contains a substitution (M to L) in the last amino acid, also represents a dominant CD8 T cell epitope for Kunjin replicon vaccines (data not shown). To analyse the levels of anti-vector CD8 T cell responses for the different vaccines shown in Fig. 3A and B, ELISPOT assays using the GYISTRVEL peptide epitope (at 10 μg/ml) were undertaken. Ex vivo IFN-γ ELISPOT analysis showed that responses ranged from ≈400 to 1000 spots/10⁶ splenocytes (Fig. 3C). Cultured IFN-γ ELISPOT analysis showed a range of ≈2000–5000 spots/10⁶ splenocytes for all the vectors except DX, which gave a large mean response in excess of 40,000 spots/10⁶ splenocytes (Fig. 3D). The very large response for DX might be due to the large number of VLPs not expressing Gag present in the DX preparation (Table 2). These results illustrate that flavivirus replicon-based vectors, like other vector systems [44,45], also induce CD8 T cell responses specific for vector proteins.

3.4. Insert stability

We and others [46] have noted that flavivirus VLPs can lose expression of certain inserted genes. To investigate to what extent the different SIV gag VLP vaccines suffered from this problem, Vero cells were infected with VLPs and dual-labelled with anti-Kunjin NS1 and anti-Gag antibodies. For WT and Gag-pol the majority of VLP-infected cells expressed both NS1 and Gag, however, for DX and OPT only about 22% and 40% of NS1 positive cells, respectively, also expressed Gag (Table 2). For the HIV gag VLPs this value was >90% (data not shown). The DX and OPT constructs contained RNA- and codon-optimised genes, perhaps suggesting that altering the coding sequence somehow reduces the stability of these inserted genes in the Kunjin replicon constructs. The in vitro transcribed RNA used to transfect the packaging cells did not contain any detectable truncated replicon RNA species (Fig. 4A) indicating that these insert deletion events likely occurred during VLP manufacture. Gene deletion events from flavivirus vectors have not been extensively studied. To gain some insight into the process, RNA from WT and DX VLP-infected cells was analysed by RT-PCR using primers flanking the SIV gag gene insert. The full-length gene is visible at ≈2000 bp, with shorter PCR products evident in both WT and DX infected cells. Gene deletions in WT and DX appear to be different, with DX showing a major band at ≈180 bp (Fig. 4B, lane 3). A band of the same size was also observed in samples from OPT-infected cells (data not shown). This ≈180 bp fragment is actually smaller than that obtained from the empty vector (Fig. 4B, lane 4),
suggested that at least one end of the deletion must lie within the ubiquitin cloning site or the FMDV 2A protease region (see Fig. 1). Sequencing of the ~180bp fragment from DX illustrated that the deletion occurred 15 nucleotides past the 5′ end of the 225 nucleotide Ub sequence and 29 nucleotides past the 5′ end of the 48 nucleotide FMDV2A sequence (Fig. 4B). There are no obvious homologies between the sequences either side of the insert deletion site that would suggest strand switching or homologous recombination events.

Table 2
Percentage of NS1-expressing cells also expressing SIV Gag following infection with SIV gag VLP vaccines

| SIVgag construct | % gag positive |
|------------------|----------------|
| WT               | 69.3 ± 0.08    |
| DX               | 22.6 ± 0.49    |
| OPT              | 40.4 ± 0.43    |
| gag-pol          | 870 ± 0.01     |

Each VLP vaccine was titrated on Vero cells and cultured for 48 h. IFA was then performed using anti-NS1 and anti-SIV Gag (5S-2F12) monoclonal antibodies and the IU titre calculated for each antibody. The ratio of the titres is represented as a percentage (Gag titre/NS1 titre × 100).

3.5. Protection assays

Recombinant vaccinia viruses encoding HIV-1 antigens have been used extensively as surrogate viral challenge models in mice to assess protective immune responses induced by different HIV-1 vaccines [11,47]. We determined whether two recombinant vaccinia viruses, one encoding SIV MNE gag/pol [48] (kind gift from Dr. Shiu-Lok Hu, University of Washington, Seattle, WA) and the other SIV mac251 gag (EVA261, Centre for AIDS Reagents, National Institute for Biological Standards and Controls, UK) could be used in such assays. Unfortunately, although they grew well in vitro, both vaccinia viruses replicated very poorly in mice following i.p., intranasal or intravenous inoculations of 10⁶ or 10⁷ pfu of virus (data not shown), making them unsuitable for such assays. A vaccinia virus encoding HIV-1 gag was run in parallel and replicated efficiently as described previously [11]. A vaccinia encoding SIVmac239 gag has been reported to replicate in mice [1], however, this vaccinia virus has not been made publicly available, and the reason for its better replication in mice is unclear.

To develop an alternative challenge assay we generated an A20 lymphoblastic leukemia line stably expressing SIV gag DX fused to EGFP (A20-EGFP-SIVgag cells). Western blotting illustrated that these cells expressed the Gag-EGFP fusion protein, which has an expected molecular weight of ~82kDa (Fig. 5A). These cells were used to challenge animals ~9 months after vaccination with the different SIV VLP vaccines. The OPT, Gag-pol and DX vaccines all provided significantly better protection than both the controls and the WT vaccine (log-rank tests all R² ≥ 0.006) (Fig. 5B). OPT vaccination also provided significantly better protection than DX (p = 0.031), with no other comparisons showing significant differences (Fig. 5B). Mean tumour growth curves from the same experiment are shown in Fig. 5C. The percentage of mice surviving after challenge with A20-EGFP-SIVgag cells (0, 33, 67 and 100% for WT, DX, Gag-pol, and OPT, respectively, Fig. 5B) correlated well with the mean ex vivo ELISPOT data shown in Fig. 3A (100, 119, 183 and 248 spots/10⁶ splenocytes, respectively), R² = 0.95. A similar comparison with the cultured ELISPOT data (Fig. 3B) failed to show any correlation, R² = 0.04, suggesting that effector memory rather than central memory cell activity had a major role in mediating protection in this model.

4. Discussion

Vaccines against a series of pathogens including HIV/SIV are being developed using a number of RNA-based viral vector systems. Here we describe the behaviour of four different Kunjin replicon VLP vaccines encoding SIV gag and show that only the Gag-pol vaccine (i) induced good levels of both effector memory and central memory T cell responses 10 weeks post-vaccination, comparable to those induced by the previously described HIV-1 gag Kunjin replicon VLP vaccine [11], (ii) showed good levels of protection against challenge with A20 cells expressing SIV Gag ~9 months post-vaccination, and (iii) displayed high levels of insert stability. WT and DX vaccines induced poor effector memory responses and provided poor protective activity, and OPT and DX showed high levels of insert deletion.

The potential for genetic instability for many recombinant viral vectors is well described and a range of largely vector-specific strategies have been adopted to limit the problem [46,49–53]. In the Kunjin replicon system deletion of inserted genes appears to occur in packaging cells during VLP manufacture. For constructs with low insert stability the proportion of VLPs without inserts increases if the time between RNA transfection of the packaging line and VLP harvest is extended, and appropriate reductions of this
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Fig. 3. T cells responses induced after immunisation with Kunjin-SIVmac239 gag VLP vaccines. (A) Measurement of Gag-specific responses by ex vivo ELISPOT assays. BALB/c mice (*n* = 3 or 4 per group) were immunised twice with 10^6 IU of each of the indicated Kunjin VLP vaccines. There were two control groups, one received 10^6 IU of a control VLP encoding an irrelevant antigen (control VLP) and the other received no vaccination (control naive). Ten weeks after the second immunisation, the mice were sacrificed and the splenocytes were assayed for Gag-specific T cell responses by ex vivo IFNγ/H9253 ELISPOT using six pools of overlapping peptides spanning either the SIV Gag protein for SIV gag vaccinated animals, or the HIV-1 Gag protein for HIV-1 gag vaccinated animals. Error bars illustrate the variation in responses to each pool. (B) Measurement of Gag-specific responses by cultured IFNγ/H9253 ELISPOT assays. The splenocytes from the animals described in (A) were also cultured for 6 days with the pooled SIV or HIV Gag peptides, prior to the ELISPOT assay. (C) Measurement of NS3-specific responses by ex vivo IFNγ/H9253 ELISPOT assays. The splenocytes from the animals described in (A) were also used in an ex vivo ELISPOT assays using the NS3 CDB T cell peptide epitope, GYISTRVEL. (D) Measurement of NS3-specific responses by cultured IFNγ/H9253 ELISPOT assays. The splenocytes from the animals described in (A) were cultured for 6 days with GYISTRVEL peptide prior to an ELISPOT assay using the same peptide.

time period can result in VLP preparations with substantially fewer empty VLPs (unpublished observation). These observations parallel those obtained for West Nile replicons where VLPs with insert deletions were also rapidly selected during serial passage of VLPs in a packaging cell line [46]. In contrast, we have generated by antibiotic selection several cell lines stably transfected with recombinant Kunjin replicon vectors and these could be extensively passaged without loss of reporter gene inserts [12], suggesting that insert deletion may be rare once a cell is transfected. One might speculate that insert deletion is associated with the cellular stress that leads to the cytopathic effects, which are observed in the packaging cells during VLP manufacture due to the high level of structural protein expression. Conceivably, in such stressed cells replicon RNA may gain access to the nuclear RNA splicing machinery. RNA splicing is well known to be influenced by complex RNA structure interactions, with distant RNA sequences able to regulate splicing events [54]. Why DX and OPT vaccines would be more susceptible to insert deletion remains unclear, but may be associated with the loss of native RNA structures.

The DX gene was engineered to allow Rev-independent expression from DNA-based vector systems [23,24], however, this did not provide significant improvements in antigen expression. The negative influence of SIV gag INS sequences on RNA stability and translation [25,26] therefore do not appear to be significant in this system. Successful expression of wild-type SIV Gag by other vector systems where mRNA is generated in the cytoplasm (e.g. alphavirus replicons and vaccinia) also supports the view that the major influence of INS elements is on RNA export [9,55]. The human codon-optimised Kunjin OPT construct did not result in significantly higher Gag expression in Vero (monkey) or BHK (hamster) cells, suggesting that tRNAs are not particularly limiting in these replicon-based expression systems when tested in vitro.

Why OPT and Gag-pol might provide slightly improved effector memory responses and WT and OPT induce such poor central memory responses when delivered by Kunjin replicon VLPs remains unclear, but is likely to be a reflection of multiple factors including the number of empty VLPs in the vaccine inocula, the level and length of antigen expression in vivo, and the level of “danger
signals” induced by the replicating replicon RNA. The better memory responses induced by the Gag-pol vaccine may be due to the removal of the potentially immunosuppressive gag-encoded proteins [29]. An alphavirus replicon SIV vaccine with these regions deleted also showed improved longevity of T cell responses over full-length gag constructs [8]. The high (albeit variable) central memory responses induced by DX may arise from increased levels of IFN-α/β produced [56] as a result of the large number of empty VLPs that compromised ≈80% of the VLP inocula for this vaccine preparation (Table 2).

The A20-EGFP-SIV gag challenge model (Fig. 5) represents a new model for testing the effectiveness of T cell induction by SIV gag vaccines. This model is likely to represent a measure of SIV Gag-specific CD8 T cell activity as rejection of this tumour has previously been shown to be CD8 T cell dependent [57]. Protection from this model also correlated well with the magnitude of ex vivo ELISPOT (effector memory) responses rather than cultured ELISPOT (central memory) responses induced by each of the Kunjin SIV vaccines. This might be expected since tumours are generally poor at stimulating the differentiation of central memory cells into effectors, so prophylactic protection would rely on effector memory cells [58]. The relative importance of effector memory and/or central memory cells for prophylactic protection against HIV-1/SIV has not been extensively studied. Some evidence suggests central memory cells are more important than effector memory cells [59], whereas others have argued that effector activity early in infection (when effector mem-

Fig. 4. Analysis of insert deletions in the Kunjin SIV gag vaccines. (A) Non-denaturing agarose gel and ethidium bromide staining of in vitro transcribed RNA for the indicated constructs prior to transfection of packaging cells and VLP manufacture. Note the double-stranded DNA markers do not accurately illustrate the size of the transcribed single-stranded RNA species, which are about 10.5 kb. (B) Vero cells were infected with WT (lane 2), DX (lane 3), empty VLPs (lane 4) or nothing (lane 5) and RT-PCR performed on extracted RNA using primers flanking the multiple cloning site region into which the gag genes were inserted for the former two constructs. Lane 1 shows the markers and lane 6 no template control. (C) Sequencing of the ≈180 bp fragment from DX transfected cells. The insert deletion is schematically represented with the sequence either side of the insert deletion site given beneath.

Fig. 5. Challenge of Kunjin SIV gag VLP vaccinated mice with A20 cells expressing SIV mac239 Gag. (A) Western blot of A20-EGFP-SIVgag and parental A20 cells with anti-SIV Gag antibody (55-2F12) and anti-GFP antibody. 15 μg of total protein was loaded in each well. (B) Kaplan Meier plot of survival. Groups of mice (n = 4–6) were immunised twice with the indicated VLP vaccines and were then challenged 9 months later with A20-EGFP-SIVgag cells. Animals were euthanased when tumours reached 100 mm². (C) Mean growth curves for the tumours from the same experiment. When an animal was euthanased a value of 100 mm² was included for that animal in the mean for all subsequent time points.

In summary we describe here a Kunjin replicon SIV Gag-pol VLP vaccine, which showed high insert stability, good induction of effector and central memory responses, and good protection against a model challenge. Other SIV gag vaccines failed in one or more of these criteria, illustrating that antigen construction and the insert’s nucleotide sequences can have unforeseen consequences for the performance of these RNA replicon vectors.

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Complete Set; SIVmac251 (BK28) Pr55 Gag.

References

[1] Nakaya Y, Zheng H, Garcia-Sastre A. Enhanced cellular immune responses to SIV Gag by immunization with influenza and vaccinia virus recombinants. Vaccine 2002;21:370–6.
[2] Nakaya Y, Nakaya T, Park MS, Cros J, Imanishi J, Palese P, et al. Induc-
tion of cellular immune responses to simian immunodeficiency virus gag by two recombinant negative-strand RNA vectors. J Virol 2004;78:9366–
75.
[3] von Messling V, Cattaneo R. Toward novel vaccines and therapies based on negative-strand RNA viruses. Curr Top Microbiol Immunol 2004;283:281–312.
[4] Griesenbach U, Inoue M, Hasagawa M, Alton EW. Sendai virus for gene therapy and vaccination. Curr Opin Mol Ther 2003;5:346–52.
[5] Eriksson KK, Makia D, Maier R, Ludewig B, Thiel V. Towards a coronavirus-based HIV multigene vaccine. Clin Dev Immunol 2006;13:353–60.
[6] Negrì DR, Micheloni S, Spada M, Vendetti S, Biffa V, et al. Successful immunization with a single injection of non-integrating lentiviral vector. Mol Ther 2007;15:1716–23.
[7] Johnston RE, Johnson PR, Connell MJ, Montefiori DC, West A, Collier ML, et al. Vaccination of macaques with SIV immunoconjugates delivered by Venezuelan equine encephalitis virus replicon particle vectors followed by a mucosal chal-
lege with SIVsmE660. Vaccine 2005;23:4969–79.
[8] Cecil C, West A, Collier M, Jurgens C, Madden V, Whitemore A, et al. Structure and immunogenicity of alternative forms of the simian immunodeficiency virus gag protein expressed using Venezuelan equine encephalitis virus replicon particles. Virology 2007;362(2):362–73.
[9] Kong W, Tian C, Liu B, Yu XF. Stable expression of primary human immunodefi-
ciency virus type 1 structural gene products by use of a noncytopathic sindbis virus vector. J Virol 2002;76:11434–9.
[10] Anderson MJ, Porter DC, Moldoveanu Z, Fletcher 3rd TM, McPherson S, Morrow RS. Vaccination preserves CD4 memory T cells during acute simian immuno-
deficiency virus SIVmac239. J Virol 2006;80:5875–85.
[11] Schwartz S, Felber BK, Pavlakis GN. Distinct RNA sequences in the gag region of simian immuno-
deficiency virus type 1 influence the capacity and genetic stability of West Nile virus replicon vectors. J Virol 2006;80:5875–85.
[12] Zolotukhin AS, Michalowski D, Bear J, Smulevitch SV, Traish AM, Peng R, et al. Effects of the human immunodeficiency virus type 1 mRNA 3'-untranslated region on regulation of expression of regulatory elements. Mol Cell Biol 2003;23:6618–30.
[13] Schwartz S, Felber BK, Pavlakis GN. Distinct RNA sequences in the gag region of simian immunodeficiency virus type 1 decrease RNA stability and inhibit expression in the absence of Rev protein. J Virol 1992;66:150–9.
[14] Liu WJ, Zhao KN, Gao FG, Leggatt GR, Fernandez J, Fahey JR. Polynucleotide viral vaccines: codon optimization and ubiquitin conjugation enhances prophylactic and therapeutic efficacy. J Virol 2005;79:8622–5.
[15] Davies MN, Flower DR. Harnessing bioinformatics to discover new vaccines. Drug Discov Today 2007;12:389–95.
[16] Bolesta E, Gyi J, Wierzbicki A, Kmieciak D, Kowalczyk A, Kaneko Y, et al. Clustered epitopes within the Gag-Pol fusion protein DNA vaccine enhance immune responses and protection against challenge with recombinant vac-
cinia viruses expressing HIV-1 Gag and Pol antigens. Virology 2005;332:467–
77.
[17] Varmaiski AN, Young PR, Khorramkhoo AH. Stable high-level expression of het-
erologous genes in vitro and in vivo by noncytopathic DNA-based Kunjin virus replicon vectors. J Virol 2000;74:4394–403.
[18] Heister, H., Kodama T, Ringer D, Marthas M, Pedersen N, Lacker M, et al. Induction of AIDS in thesus monkeys by molecularly cloned simian immunodefi-
ciency virus. Science 1999;284:1109–12.
[19] Negrì DR, Buffa V, Leone P, Bona R, Bortghi M, Carlini F, et al. Use of retroviral vector-based HIV-1 vaccine and therapeutic DNA vaccines for the induction of a protective immune response in chimpanzees. Vaccine 2006;24:1378–88.
[20] Westaway EG, Mackenzie IM, Kenney MT, Jones MK, Khorramkhoo A. Ultra-
structure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J Virol 1997;71:6650–61.
[21] Macdonald J, Toney J, Hall RA, Williams B, Palacios G, Ashok MS, et al. NS1 protein secretion during the acute phase of West Nile virus infection. J Virol 2005;79:13924–33.
[22] Miroux B, Walker JE. Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 1996;260:289–98.
[23] Elliott SL, Pye S, Le T, Mateo L, Cox J, Cotrell I, Pearson M, et al. Cytotoxic T cell polyepitope vaccines delivered by ISCOMs. Vaccine 2001;19:4669–75.
[24] Elliot SL, Pye S, Le T, Mateo L, Cox J, Macdonald L, et al. Peptide based cyto-
toxic T-cell vaccines; delivery of multiple epitopes, help, memory, and problems. Vaccine 1999;17:2009–19.
[25] Someya K, Kin KQ, Matsu K, Okuda K, Yamamoto N, Honda M. A consecu-
tive priming–boosting vaccination of mice with simian immunodeficiency virus (SIV) gag/pol DNA and recombinant vaccinia virus strain D6 elicits effective SIV immunity. J Virol 2004;78:9842–52.
[26] Barouch DH, Pau MG, Custers JH, Koustaal W, Kostense S, Havenga MJ, et al. Immunogenicity of alternative forms of the simian immunodeficiency virus (SIV) gag/pol DNA and recombinant vaccinia virus strain D6 elicits effective SIV immunity. J Virol 2004;78:9842-52.
[27] Westaway EG, Mackenzie IM, Kenney MT, Jones MK, Khorramkhoo A. Ultra-
structure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J Virol 1997;71:6650–61.
[49] Kriajevska MV, Zakharova LG, Altstein AD. Genetic instability of vaccinia virus containing artificially duplicated genome regions. Virus Res 1994;31:123–37.

[50] Junker U, Bohnlein E, Veres G. Genetic instability of a MoMLV-based antisense double-copy retroviral vector designed for HIV-1 gene therapy. Gene Ther 1995;2:639–46.

[51] Lee SG, Kim DH, Hyun BH, Bae YS. Novel design architecture for genetic stability of recombinant poliovirus: the manipulation of G/C contents and their distribution patterns increases the genetic stability of inserts in a poliovirus-based RPS-Vax vector system. J Virol 2002;76:1649–62.

[52] Dufresne AT, Dobrikova EY, Schmidt S, Gromier M. Genetically stable picornavirus expression vectors with recombinant internal ribosomal entry sites. J Virol 2002;76:8966–72.

[53] Raju R, Subramaniam SV, Hajjou M. Genesis of Sindbis virus by in vivo recombination of nonreplicative RNA precursors. J Virol 1995;69:7391–401.

[54] Valadkhan S. The spliceosome: caught in a web of shifting interactions. Curr Opin Struct Biol 2007;17:310–5.

[55] Swanson CM, Puffer BA, Ahmad KM, Domas RW, Malim MH. Retroviral mRNA nuclear export elements regulate protein function and virion assembly. EMBO J 2004;23:2632–40.

[56] Mescher MF, Curtisinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, et al. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev 2006;211:81–92.

[57] Liu A, Guardino A, Chinsangaram L, Goldstein MJ, Panicali D, Levy R. Therapeutic vaccination against murine lymphoma by intratumoral injection of recombinant fowlpox virus encoding CD40 ligand. Cancer Res 2007;67:7037–44.

[58] Fuchs EJ, Matzinger P. Is cancer dangerous to the immune system? Semin Immunol 1996;8:271–80.

[59] Vaccari M, Trindade CJ, Venzon D, Zanetti M, Franchini G. Vaccine-induced CD8+ central memory T cells in protection from simian AIDS. J Immunol 2005;175:3502–7.

[60] Altes HK, Price DA, Jansen VA. Effector cytotoxic T lymphocyte numbers induced by vaccination should exceed levels in chronic infection for protection from HIV. Vaccine 2001;20:3–6.

[61] Jansen CA, van Baarle D, Medema F. HIV-specific CD4+ T cells and viremia: who’s in control? Trends Immunol 2006;27:119–24.

[62] Brown K, Gao W, Alber S, Trichel A, Murphey-Corb M, Watkins SC, et al. Adenovirus-transduced dendritic cells injected into skin or lymph node prime potent simian immunodeficiency virus-specific T cell immunity in monkeys. J Immunol 2003;171:6875–82.

[63] Brant D, Blissenbach M, Grew B, Konietzny R, Grunwald T, Uberla K. Rev proteins of human and simian immunodeficiency virus enhance RNA encapsidation. PLoS Pathog 2007;3:e54.

[64] Zhu Y, Koo K, Braddock JD, Sutton WF, Koller LR, Bucaii R, et al. Macaque blood-derived antigen-presenting cells elicit SIV-specific immune responses. J Med Primatol 2000;29:182–92.

[65] Brant D, Grunwald T, Lucie S, Stang A, Uberla K. Functional replacement of the R region of simian immunodeficiency virus-based vectors by heterologous elements. J Gen Virol 2006;87:2297–307.

[66] McDonald D, Vodicke MA, Lucero G, Svitkina TM, Borisy GG, Emerman M, et al. Visualization of the intracellular behavior of HIV in living cells. J Cell Biol 2002;159:441–52.

[67] Martin-Serrano J, Zang T, Bieniasz PD. HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. Nat Med 2001;7:1313–9.

[68] Pfeiffer T, Pisch T, Devitt G, Holtkotte D, Bosch V. Effects of signal peptide exchange on HIV-1 glycoprotein expression and viral infectivity in mammalian cells. FEBS Lett 2006;580:3773–8.

[69] Moses AV, Stenglein SG, Strussenberg JG, Wherly K, Chesebro B, Nelson JA. Sequences regulating tropism of human immunodeficiency virus type 1 for brain capillary endothelial cells map to a unique region on the viral genome. J Virol 1996;70:3401–6.