Kunjin Virus Replicon Vaccine Vectors Induce Protective CD8+ T-Cell Immunity

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The ability of self-replicating RNA (replicon) vaccine vectors derived from the Australian flavivirus Kunjin (KUN) to induce protective αβ CD8+ T-cell responses was examined. KUN replicons encoding a model immunogen were delivered by three different vaccine modalities: (i) as naked RNA transcribed in vitro, (ii) as plasmid DNA constructed to allow in vivo transcription of replicon RNA by cellular RNA polymerase II (DNA based), and (iii) as replicon RNA encapsidated into virus-like particles. A single immunization with any of these KUN replicon vaccines induced CD8+ T-cell responses at levels comparable to those induced by recombinant vaccinia virus encoding the same immunogen. Immunization with only 0.1 μg of DNA-based KUN replicons elicited CD8+ T-cell responses similar to those seen after immunization with 100 μg of a conventional DNA vaccine. Naked RNA immunization with KUN replicons also protected mice against challenges with recombinant vaccinia virus and B16 tumor cells. These results demonstrate the value of KUN replicon vectors for inducing protective antiviral and anticancer CD8+ T-cell responses.

Replicon-based vectors of positive-strand RNA viruses are becoming more and more popular for development of antiviral and anticancer vaccines (reviewed in reference 24). Several features make these vectors a desirable choice for development of highly efficient and safe vaccines. These include (i) a high level of expression of encoded heterologous genes (HGs) due to the ability of replicon RNA to amplify itself; (ii) exclusive cytoplasmic replication, which eliminates any possible complications associated with nuclear splicing and/or chromosomal integration; (iii) inability of the replicon RNA to escape from the transfected (or infected) cell, thus limiting the spread of the vaccine vector in the immunized subject, which makes these vectors biologically safe; and (iv) relatively small genome size (7 to 9 kb), allowing easy manipulations with the cDNA and generation of recombinants. An additional beneficial feature of replicon vectors is a variety of modalities that can be used to deliver replicon RNA into cells (Fig. 1A) (24). It can be either delivered directly as naked RNA transcribed in vitro (RNA based), packaged first into virus-like particles (VLPs) and then delivered via infection with these VLPs, or delivered in the form of plasmid DNA, which encodes replicon cDNA placed under the control of a mammalian expression promoter (DNA based), allowing the production of functional replicon RNA in vivo by the cellular RNA polymerase II (Fig. 1A). Replicon-based expression vectors have been developed for representatives of most positive-strand RNA virus families, including alphaviruses, picornaviruses, and flaviviruses (reviewed in reference 24). However, the great majority of the data on immunogenic properties of replicon vectors in laboratory animals have been accumulated using replicons of alphaviruses such as Sindbis virus (SIN), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE) (see reference 47 for a review and references 8, 10, 11, and 39 for more recent studies). In general, these studies showed that alphavirus replicon vectors induced strong antibody and CD8+ T-cell responses to encoded immunogens and in most cases protected immunized animals from appropriate virus or tumor challenges. All three delivery modalities were effective; however, VLP delivery was shown to be the most efficient. In comparative studies of conventional (nonreplicating) plasmid DNA vectors and alphavirus DNA-based replicon vectors, the latter generally induced stronger immune responses and at significantly lower DNA concentrations than did conventional vectors (4, 17). However, in some studies the efficiency of immune responses induced by DNA-based alphavirus replicons appeared to depend on the nature of the encoded immunogen, and the immunity to some immunogens was shown to be similar to or lower than that induced by the same amount of conventional plasmid DNA vector (10, 22).

We have been developing replicon vectors derived from the flavivirus Kunjin (KUN) (25, 27, 52, 53). The KUN replicon expression system, like alphavirus replicon systems, allows delivery of replicon RNA by three different modalities, i.e., as naked RNA, as VLPs, and as plasmid DNA (Fig. 1A) (24, 52, 53). Unlike homologous alphavirus replicon packaging systems (6, 33, 41), the KUN replicon packaging system makes use of a replicon expression vector derived from the unrelated SFV for production of KUN structural proteins (27, 52). This eliminates any potential problems associated with contamination of VLPs with infectious recombinant material. Additionally, in contrast to highly cytopathic alphavirus replicon vectors commonly used in immunization studies, KUN replicons appear to be noncytopathic and allow prolonged expression of HGs both...
Although noncytopathic alphavirus replicon vectors have been developed (1, 12, 40), no data on their immunogenic properties have been reported. In our previous studies we demonstrated that immunizations of mice with a noncytopathic DNA-based KUN replicon vector expressing the \( \beta \)-galactosidase gene resulted in induction of strong anti-\( \beta \)-galactosidase antibody responses (53). Here we show that immunization of mice with KUN replicons expressing the murine polyepitope or polytope (Mpt) (50, 51) as a model immunogen and delivered in the form of naked RNA, VLPs, or plasmid DNA resulted in induction of CD8\(^+\) T-cell responses specific to encoded epitopes and protected mice from viral and tumor challenges.

**MATERIALS AND METHODS**

**Plasmids.** RNA-based (RNALeu) and DNA-based (DNALeu) KUN replicon vectors contain two copies of 2A autoprotease of the foot-and-mouth disease virus (FMDV2A), one upstream and another downstream of the Mpt sequence. Pro and Leu variants contain amino acid Pro or Leu at position 250 in the KUN NS1 gene product, respectively.
DNA and RNA transfections, 1F, and Northern blot analyses. For DNA transfections, BHK21 cells in six-well plates or on coverslips were transfected with 2 or 0.4 μg of plasmid DNAs, respectively, by using Lipofectamine Plus transfection reagent (Life Technologies, Melbourne, Australia), as described by the manufacturer. RNAs were transfected into vitro by the Sp6 RNA polymerase from the Xhol-linearized RNA-based plasmid DNAs and electroporated into BHK21 cells as described previously (25). Coverslips with transfected cells were fixed in cold acetone at 48 h after transfections and assayed for expression of KUN NS3 protein by indirect immunofluorescence (IF) with anti-NS3 antibodies as described previously (56). Northern blot analysis of total RNA from transfected cells was performed as described previously (25) using 32P-labeled cDNA probes representing either the KUN 3′ untranslated region (UTR) or the Mpt sequence.

Preparation of VLPs. VLPs (VLPProMpt) were prepared as described previously (27, 52). Briefly, 2 × 10⁵ BHK21 cells were electroporated with −10 to 20 μg of in vitro-transcribed RNAProMpt RNA at 1.5 kV, 25 μF, and 0% resistance, with two pulses at a 10-s interval. After electroporation cells were diluted in 8 ml of Dulbecco modified Eagle medium (DMEM)−10% fetal calf serum (FCS) and cultured in 60-mm-diameter dishes at 37°C in a CO₂ incubator. After 32 h cells were trypsinized and used for a second electroporation with in vitro-transcribed SFV replicon RNA expressing KUN structural proteins (SFV-MEC105) (27) under the same conditions. At 18 h after the second electroporation, the medium was replaced with 3 ml of DMEM−2% FCS, and cells were incubated for a further 22 h. The medium was harvested and clarified by spinning at 8,000 × g prior to storage in 1-ml aliquots at −80°C. The titer of infectious VLPs was determined by infecting BHK21 cells with 10-fold serial dilutions of the VLPs and counting the number of NS3-positive cells after IF analysis at 30 to 40 h postinfection.

Immunization of mice. Female BALB/c (H-2d) mice (6 to 8 weeks) were supplied by the Animal Resources Centre (Perth, Western Australia, Australia). Mice were immunized with the following formulations: (i) KUN replicon polytope DNA plasmids, DNALeuMpt and DNAProMpt, encoding KUN replicon expressing Mpt and injected into the quadriceps muscles (100 μg in 100 μl of phosphate-buffered saline [PBS], intramuscularly [i.m.], 50 μl into each leg); (ii) in vitro-transcribed KUN replicon RNA, RNALeuMpt and RNAProMpt, encoding Mpt, dissolved in diethyl pyrocarbonate-treated PBS and injected as described above (−30 μg in 100 μl, i.m., 50 μl in each leg); (iii) replicon RNA RNAProMpt packaged into VLPs (VLPProMpt) delivered in DMEM−2% FCS and injected intraperitoneally (i.p.) (−5 × 10⁸ VLPs in 1 ml); (iv) a conventional plasmid DNA vaccine, pSTMPDV, encoding Mpt (100 μg in 100 μl of PBS, i.m., 50 μl into each quadriceps muscle) (50); and (v) a recombinant vaccinia virus encoding Mpt (10⁵ PFU in 200 μl of RPMI 1640, i.p.), as described previously (51).

CD8 T-cell assays. Epitope-specific gamma interferon (IFN-γ)-secreting cells were enumerated by an enzyme-linked immunospot (ELISPOT) assay with minimal CD8 T-cell peptide epitopes as described previously (30). Briefly, flat-bottomed 96-well Multiscreen-RA cellusose ester membrane microtiter plates (Millipore Australia Ltd., North Ryde, Australia) were coated overnight with 5 μg of rat anti-mouse IFN-γ antibody (clone RA-82, BD PharMingen, San Diego, Calif.)/ml. Coated plates were then blocked with 1% bovine serum albumin in PBS for 1 h at room temperature and washed three times with PBS containing 0.05% Tween 20 (Sigma). Splenocytes (10⁶/well) were plated in the first wells of the ELISPOT plate and serially diluted twofold. Recombinant human interleukin-2 (kindly provided by Cetus Corp., Emeryville, Calif.) (100 IU/ml) was added with peptide (Mimotopes, Clayton, Victoria, Australia) (1 μg/ml), and the plates were incubated for 18 h. The cells were lysed, the plates were washed, and IFN-γ spots were detected by incubation first with biotinylated anti-mouse IFN-γ antibody (clone XMG 1.2) (BD PharMingen) and then with streptavidin-alkaline phosphatase (BD PharMingen) and Sigma Fast 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (CBP-NBT) substrate (Sigma). Spots were counted using a KS ELISPOT reader (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). The different immunization modalities were compared using a two-way analysis of variance (ANOVA) with a model that contained the modality and all the epitope responses and used a Tukey post hoc test for multiple comparisons.

51Chromium (51Cr) release assays were performed as described previously (9). Briefly, mice were sacrificed 2 to 3 weeks postimmunization with VLPMpt, and splenocytes were restimulated in vitro for 6 days by the addition of 1 μg of peptide (20 μl). The results were expressed as the percentage of specific radioactivity released in relation to the total radioactivity added to the reaction mixtures. The experiment was done with triplicates, and the data were evaluated by a two-way ANOVA (45). The time when animals were sacrificed was also noted and represented as Kaplan-Meier survival curves (31). Differences between Kaplan-Meier survival curves were calculated using the log-rank statistic (31).

RESULTS
Efficient replication of KUN replicons encoding Mpt in transfected cells. Plasmid DNAs DNALeuMpt and DNAProMpt encoding KUN replicon cDNAs expressing Mpt were transfected into BHK21 cells, and replication and expression of corresponding replicon RNAs transcribed in cells by RNA polymerase II were examined by Northern blotting and IF analyses. In vitro-synthesized Mpt-encoding KUN replicon RNAs RNALeuMpt and RNAProMpt were transfected into BHK21 cells by electroporation and analyzed for their replication and expression. IF analysis of transfected cells with KUN anti-NS3 antibodies showed that ~50% of cells were positive after transfection with DNA-based replicons and ~80% of cells were positive after electroporation with RNA-based replicons (Fig. 2A). In our previous experiments with electroporation of KUN replicon RNAs, expression of KUN NS3 could be detected by IF only when these RNAs were capable of replication (25, 26). Transfection of plasmid DNA encoding KUN cDNA did, however, result in detection of expression of KUN proteins by IF, even when the KUN RNA was unable to replicate (29). To ensure that replicon RNAs produced in cells from transfected plasmid DNAs DNALeuMpt and DNAProMpt were replicating, we analyzed total cellular RNA by Northern blot analysis with a KUN-specific radiolabeled cDNA probe (Fig. 2A). Comparison of the intensity of the radioactive signal in these samples with that detected in the RNA sample isolated from cells transfected with the same amount of the control plasmid DNA pHKUNrep2 (dGDD) producing replication-deficient KUN replicon RNA (53) showed that ~5- to 10-fold-higher amounts of KUN-specific RNAs were produced in DNALeuMpt- and DNAProMpt-transfected cells than in pHKUNrep2 (dGDD)-transfected cells. Previous studies with replication-competent and replication-deficient KUN DNA-based replicon constructs showed similar differences in the amounts of accumulated RNAs (53). These results demonstrated that replicon RNAs

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produced in cells from the transfected plasmid DNAs DNALeuMpt and DNAProMpt were replication competent. Northern blot analysis with a KUN-specific cDNA probe of total RNA isolated from cells electroporated with in vitro-synthesized RNAs RNALeuMpt and RNAProMpt (Fig. 2B) confirmed the IF results (Fig. 2A), and together they demonstrated efficient replication and accumulation of KUN replicon RNA in transfected cells. Note that two times more total cellular RNA was loaded on the gel for RNA-transfected samples than for DNA-transfected samples (Fig. 2B) and also that ethidium bromide staining of the gel showed a similar concentration of rRNAs in corresponding (RNA-transfected or DNA-transfected) samples (data not shown).

To examine whether the Mpt sequence was retained during replication of replicon RNAs in cells, the membrane containing the same RNA samples (left panel in Fig. 2B) was reprobed with a radiolabeled cDNA probe specific for the Mpt sequence. The results (right panel in Fig. 2B) were identical to those obtained using the KUN-specific probe (left panel in Fig. 2B) and clearly demonstrated that the Mpt sequence was indeed retained during amplification of KUN replicon RNAs in transfected cells.

The purpose of generating Leu- and Pro-containing constructs was to evaluate a possible effect of this mutation at amino acid position 250 in the KUN NS1 gene product on the replication and accumulation of KUN replicon RNA and thus on the levels of expression of encoded HGs both in vitro and in vivo. It was previously shown that a Pro (wild type)-to-Leu mutation at amino acid 250 in the KUN NS1 gene product resulted in the loss of dimerization of the NS1 protein. This caused a delay in virus replication in Vero cells and attenuation of virus replication in mice (16). Infection of Vero cells with equal amounts of Leu- and Pro-containing viruses produced ~100-fold less of the Leu-containing virus in the first 24 h, but similar yields of Leu- and Pro-containing viruses were obtained by 48 h after infection. In vivo experiments, ~100-

![Image of Northern blot analysis](http://jvi.asm.org/)
fold-more Leu-containing virus was required to generate similar clinical symptoms of the disease in mice (16). In the present study with KUN-Mpt replicon constructs, the amounts of KUN RNA accumulated in BHK21 cells transfected with DNA-based Leu- and Pro-containing replicon constructs were similar (compare DNALeuMpt and DNAProMpt lanes in Fig. 2B), while transfections with in vitro-transcribed RNAs showed slightly higher levels of RNA accumulation in cells transfected with Leu-containing RNA than in cells transfected with Pro-containing RNA (compare RNALeuMpt and RNAProMpt in Fig. 2B). As mentioned above, the amounts of rRNAs were similar in corresponding samples as judged by ethidium bromide staining. Transfection of RNALeuMpt RNA also resulted in a slightly higher proportion of NS3-positive cells in IF analysis (Fig. 2A). Noticeably, a significantly higher number of rounded (dead) NS3-positive cells were seen after transfection with RNAProMpt RNA than after transfection with RNALeuMpt RNA (Fig. 2A), suggesting higher cytopathicity of the former RNA. A similar difference in cytopathicity between Pro- and Leu-containing variants was also observed following transfections of corresponding KUN replicon vector RNAs RNAPro and RNALeu (data not shown).

**Induction of CD8^+ T-cell responses following immunization with DNA-, RNA-, and VLP-based KUN replicons expressing a polytope immunogen.** The Mpt immunogen contains four conjoined CD8^+ T-cell epitopes restricted by H-2^d^; these include YPHFMPTNL (an H-2L^d^-restricted epitope from murine cytomegalovirus pp89), RPQASGVYM (H-2L^d^-restricted epitope from lymphocytic choriomeningitis virus nucleoprotein), TYQRTRALV (H-2K^d^ epitope from influenza virus nucleoprotein), and SYIPSAEKI (H-2K^d^ epitope from Plasmodium berghei circumsporozoite protein). BALB/c mice were immunized once with KUN replicon DNA, RNA, and VLPs encoding the Mpt immunogen, and after 2 to 3 weeks CD8^+ T-cell responses to each of the four CD8^+ T-cell peptide responses to each of the four CD8^+ T-cell peptide epitopes were measured using IFN-γ ELISPOT (Fig. 3A). The ELISPOT responses generated by the RNA and VLP KUN vaccines were comparable to those induced by a recombinant vaccinia virus encoding the same polytope immunogen (rVVMpt) (Fig. 3A). ANOVA showed a P value of 0.34 for RNAProMpt versus rVVMpt immunizations and a P value of 1 for VLPProMpt versus rVVMpt immunizations. A single immunization with DNAProMpt or DNALeuMpt also induced responses comparable with those reported for two immunizations with a con-

![Figure 3](http://jvi.asm.org/)

**FIG. 3.** Cytotoxic T-lymphocyte responses specific for YPHFMPTML (YPH), RPQASGVYM (RPQ), TYQRTRALV (TYQ), and SYIPSAEKI (SYI) epitopes induced by immunization with various KUN replicon-based vectors encoding Mpt immunogen. (A) ELISPOT analysis of BALB/c (n = 4 per group) mouse splenocytes following immunization with the indicated vaccines. (B) ^51Cr release assay of restimulated splenocyte populations from BALB/c mice (n = 3 per group) immunized with VLPProMpt. A standard 6-h Cr release assay was performed using labeled target cells sensitized with (filled squares) or not sensitized with (empty squares) the indicated peptides. SE, standard error.
CONVENTIONAL DNA encoding the same immunogen (30, 50) (see also Fig. 4). The hierarchy of epitope responses, with the RPO-specific responses dominating the YPH responses and the SYI responses dominating the TYQ responses, has been observed previously and is thought to reflect the lower major histocompatibility complex-binding affinity of the subdominant epitopes (30). Although Pro-containing replicons appeared to be more cytopathic than Leu-containing replicons in transfected BHK21 cells (see above), no significant differences between these variants were observed in induction of CD8⁺ T-cell responses (Fig. 3A) (ANOVA, P = 0.92 for DNAProMpt versus RNAProMpt, and P = 0.39 for DNAProMpt versus DNA LeuMpt) (see also Fig. 4).

Mice immunized once with VLPProMpt were also assayed for the induction of cytotoxic CD8⁺ T-cell responses by ⁵¹Cr release assay. Significant cytotoxic activity specific for each epitope was observed (Fig. 3B), although TYQ responses were subdominant as shown previously (30, 50, 51). These responses were again comparable with those observed previously for mice immunized once with rVV Mpt (51) and higher than those seen previously for mice immunized twice with a conventional DNA vaccine encoding the polytope immunogen (50).

Immunization of mice with 10-fold serial dilutions of the DNA-based KUN-polytope replicons DNAleuMpt and DNAProMpt illustrated that CD8⁺ T-cell responses were still detectable after a single immunization with only 0.1 µg of KUN replicon DNA (Fig. 4). The magnitude of CD8⁺ T-cell responses induced by 0.1 µg of the KUN vaccine was comparable to the magnitude of those induced by immunization with 100 µg of conventional plasmid DNA-polytope vaccine (Fig. 4) (Student’s t test results for DNAleuMpt and DNAProMpt [0.1 µg] versus conventional DNA [100 µg] were P = 0.34 and 0.55, respectively). This number of CD8⁺ T cells induced by 100 µg of KUN Mpt DNA was also approximately five to six times higher than the number induced by 100 µg of the conventional plasmid DNA-polytope vaccine (Fig. 4) (Student’s t test results for DNAleuMpt and DNAProMpt [100 µg] versus conventional DNA [100 µg] were P < 0.001 for both). The data presented in this section illustrate that KUN replicon-based vaccines, delivered by any of the three different modalities (DNA, RNA, and VLPs), can efficiently induce CD8⁺ T cells, generating responses similar in magnitude to those for recombinant vaccinia virus-based vectors and significantly higher than those for conventional DNA vaccines.

Naked RNA immunization with KUN replicon vectors encoding polytope protects mice from viral and tumor challenges. To determine whether the CD8⁺ T-cell responses induced by immunization with KUN replicon vectors could protect against viral challenge, mice were vaccinated once with RNAleuMpt RNA or RNAleuControl RNA (prepared from the RNAleu vector DNA) and were then challenged with rVV Mpt. The rVV Mpt challenge assay measured protection mediated through all four H-2d-restricted epitopes presented by the KUN vaccine. Following a single vaccination with RNAleuMpt RNA, a significant (~70%) reduction in ovari rVV titers was observed (Fig. 5A; P = 0.03). Comparable protection was obtained using a conventional DNA-polytope vaccine but only after two immunizations (50).

The Mpt sequence also encodes the ovalbumin-derived CD8⁺ T-cell epitope SIINFEKL (50, 51), permitting examination of tumor protection following KUN vaccination using challenge with B16 tumor cells expressing ovalbumin (B16-OVA). Mice were immunized twice with RNAleuMpt and RNAleuControl or once with rVV Mpt and then challenged with B16-OVA. A significantly lower rate of tumor growth was observed for RNAleuMpt-immunized mice than for RNAleu Control-immunized animals (P < 0.001) (Fig. 5B, upper graph). Furthermore, the average rate of tumor growth in RNA LeuMpt-immunized mice was also lower than that following immunization with rVV Mpt (P = 0.001). The data from the same experiment are also presented as Kaplan-Meier curves (Fig. 5B, lower graph), which show the percentage of mice surviving at the indicated time points. Again, RNAleuMpt-immunized mice performed significantly better than RNAleu Control-immunized animals (P = 0.015) and than rVV Mpt-immunized animals (P = 0.016).

DISCUSSION

We have demonstrated the ability of KUN replicon vectors to induce CD8⁺ T-cell responses to an encoded model immunogen after a single immunization. All three delivery modalities, i.e., naked RNA, plasmid DNA, and VLPs, efficiently induced CD8⁺ T-cell responses. Immunization with naked replicon RNA also protected mice from virus and tumor challenges. These results demonstrate the potential of KUN replicon-based vaccine vectors for the induction of protective CD8⁺ T-cell responses. The magnitude of CD8⁺ T-cell responses obtained using KUN replicon vectors was comparable to the magnitude of those obtained with recombinant vaccinia
virus, a vaccine vector modality widely regarded as an effective vector for CD8\(^+\) T-cell induction (44). A number of poxvirus vaccine vectors are currently being tested for their ability to induce protective CD8\(^+\) T-cell responses in primates and humans; these include modified vaccinia virus Ankara (47), fowlpox virus (43), and canarypox virus (15). Alphavirus replicons have been extensively used for the induction of immune responses in animal models, and replicons derived from VEE have recently been approved for preclinical trials in humans (http://www.alphavax.com/pp_inhouse.html). Similar to the alphavirus replicon systems, KUN replicons can be delivered by three different modalities: as naked RNA, as plasmid DNA, and as VLPs. In accordance with the results obtained with alphavirus DNA-based replicons (4, 17), immunization with only 0.1 \(\mu\)g of KUN DNA-based replicon was sufficient to elicit CD8\(^+\) T-cell responses. A similar level of CD8\(^+\) T-cell induction was achieved only after immunization with a 1,000-fold-higher dose (100 \(\mu\)g) of a conventional plasmid DNA vaccine (Fig. 4).

Naked RNA immunization is becoming increasingly popular for demonstrating the potential of replicon-based vectors to induce protective antiviral and antitumor immune responses in animal models (11, 55, 58). RNA immunization avoids issues relating to DNA integration and may offer additional advantages over immunization with VLPs. In contrast to naked RNA, immunization with VLPs is likely to lead to the induction of neutralizing antibody responses to the structural proteins of VLPs, a phenomenon which may limit the number of times that the VLPs can be used in one individual. In contrast, preexisting CD8\(^+\) T cells specific for the vaccine vector or the vaccine antigen do not normally appear to influence the efficiency of induction of CD8\(^+\) T-cell response to the vaccine antigen following immunization (48). RNA-based vaccines are also easier to manufacture than are VLPs. Recent reports also suggest that RNA can be encapsulated into microparticles and delivered into animals by bombardment with a gene gun (37, 54). Encapsulated RNA was also shown previously to be stable for 8 to 12 months at 4°C (37). Previous studies with naked RNA immunization of SIN and SFV replicon vaccines have shown effective induction of antigen-specific antibody and CD8\(^+\) T-cell responses (11, 55, 58). Similarly, we have shown that RNA immunization with KUN replicon vaccines resulted in the induction of CD8\(^+\) T-cell responses specific for an encoded immunogen with efficiency comparable to that obtained after immunizations with DNA-based or VLP-based KUN replicons (Fig. 3A). RNA vaccination using KUN or SIN replicons also induced significant antitumor protection in different tumor models (Fig. 5B) (58).

One particular feature of flaviviruses may prove to be beneficial for the use of their replicons as safe vaccine vectors. Flaviviruses, in general, have shown no evidence of recombination between different viruses or virus strains in nature. Our previous numerous complementation experiments with defective full-length KUN RNAs and replicon RNA as a helper also failed to show any evidence of recombination between these two (full-length and replicon) RNAs coreplicating in the same cell despite the presence of extended regions of perfect homology in both RNAs (summarized in reference 28). Also, no recombinations leading to the recovery of recombinant viruses were detected in complementation experiments with yellow fever virus RNAs containing deletions in the NS1 gene when the helper NS1 protein was supplied either from homologous viruses (yellow fever virus) or from heterologous viruses (dengue virus) (34, 35). This feature of flaviviruses is in contrast to the well-documented recombination abilities of alphavirus RNAs (18, 42, 46) and may provide an extra level of safety for vaccinations of individuals, who may be infected with the same...
or other flaviviruses either just before or some time after immunization. In addition, the heterologous nature of the KUN replicon packaging system, employing a replicon RNA-based vector from a totally unrelated virus, SFV, for the expression of KUN structural proteins, as well as the specific design of the expression construct, makes KUN VLP preparations absolutely safe and free of any infectious recombinant viral material (27, 52).

Perhaps one of the most distinctive features of the KUN replicons is that their replication does not cause an overt cytopathic effect or apoptosis (25, 52, 53). Cytopathic effect or apoptosis of infected cells containing vaccine antigens was reported elsewhere to be an efficient method for cross priming, resulting in the delivery of vaccine antigens to dendritic cells (DCs) and effective CD8+ T-cell induction (2, 58). However, despite exhibiting a very low or undetectable level of cytopathicity, KUN replicons with limited levels of cytopathicity may allow prolonged antigen induction (26). Furthermore, no significant differences were observed in the inductions of CD8+ T-cell responses by the Leu- and Pro-containing variants of the KUN replicon vectors (Fig. 3 and 4). However, notwithstanding elsewhere to be an efficient method for cross priming, KUN replicons efficiently induced CD8+ T-cell responses in mice (Fig. 3 and 4). Furthermore, no significant differences were observed in the inductions of CD8+ T-cell responses by the Leu- and Pro-containing variants of the KUN replicon vectors (Fig. 3 and 4), which had shown different degrees of cytopathicity in transfected BHK21 cells (Fig. 2A). CD8+ T-cell induction by KUN replicons may thus be independent of apoptosis-mediated cross priming (5) and/or may not require cross priming. It has been shown elsewhere that flaviviruses can directly infect, replicate in, and activate DCs (19–21, 32, 57), suggesting that KUN replicons may also be able to replicate and produce vaccine antigens in DCs. Direct infection of DCs by vaccine vectors may represent a more efficient strategy for CD8+ T-cell induction than cross priming (49). Lymphotrophic VEE replicon particles as well as DC-adapted SIN replicon particles were recently shown elsewhere to be able to infect DCs and express an encoded vaccine antigen (14, 36). However, the cytopathic nature of alphavirus replicon RNA may lead to apoptosis of antigen-presenting DCs and a reduction in CD8+ T-cell activation or induction. In contrast, KUN replicons with limited levels of cytopathicity may allow prolonged antigen expression in DCs without causing apoptosis, a feature which may contribute to prolonged stimulation of CD8+ T-cell responses. Induction of tolerance is unlikely, due to the ever-present double-stranded RNA, which is believed to induce danger signals and activate DCs (13).

In summary, KUN replicons have been shown to be effective vaccine vectors for the induction of protective CD8+ T cells and may represent an attractive new modality for cancer and human immunodeficiency virus vaccines. We are currently exploring the ability of these persistent KUN vaccines to maintain long-term effector CD8+ T-cell populations, a feature that may be highly desirable for prophylactic human immunodeficiency virus vaccines, as memory CD8+ T-cell responses may be insufficient to protect against challenge (23, 38).

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