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Preliminary Evaluation of a Bunyavirus Vector for Cancer Immunotherapy

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Replicon particles of Rift Valley fever virus, referred to as nonspreading Rift Valley fever virus (NSR), are intrinsically safe and highly immunogenic. Here, we demonstrate that NSR-infected human dendritic cells can activate $CD8^+$ T cells *in vitro* and that prophylactic and therapeutic vaccinations of mice with NSR encoding a tumor-associated CD8 peptide can control the outgrowth of lymphoma cells *in vivo*. These results suggest that the NSR system holds promise for cancer immunotherapy.

Dendritic cells (DCs) are the most potent antigen-presenting cells in the body and are instrumental in directing adaptive immune responses against pathogens and tumors. DCs are naturally targeted by many arboviruses. Infection induces DC maturation and presentation of virus-associated antigens, explaining the interest in using these viruses as therapeutic vectors of tumor-associated antigens. Members of the positive-strand RNA virus families *Togaviridae* and *Flaviviridae* have been extensively evaluated for cancer immunotherapy (1–4), some of which have already entered clinical trials (2). Remarkably, arboviruses with segmented negative-strand RNA genomes have remained largely unexplored in this field. The genome segments of these viruses form double-stranded RNA panhandles that, in some viruses, contain a 5′ triphosphate. This structure is an optimal ligand for the cytoplasmic pattern recognition receptor RIG-I and thereby an excellent inducer of adjuvanting innate immune responses (5). We therefore propose that arboviruses with segmented negative-strand RNA genomes that activate RIG-I hold promise for cancer immunotherapy.

Rift Valley fever virus (RVFV), a member of the *Bunyaviridae* family, was recently shown to productively infect immature DCs with high efficiency (6). Whereas wild-type RVFV counteracts innate immune responses via the NSs protein (7), infection of DCs with RVFV variants lacking the NSs protein was shown to result in strong interferon (IFN) responses triggered by RIG-I signaling (8). We recently developed RVFV particles that lack not only the gene for NSs but also the glycoprotein-encoding genome segment (9). The resulting nonspreading RVFV (NSR) particles are unable to produce progeny virions, ensuring their safe application (9). Moreover, the absence of the gene for NSs provides a slot for the expression of a gene of interest. Vaccination with NSR particles was shown to be highly effective in protecting livestock from RVFV (10–12), and NSR particles expressing the influenza virus hemagglutinin gene protected mice from lethal influenza (13). The remarkable efficacy of NSR-based vaccines in both inbred and outbred animals, particularly its association with Th1-type immune responses (13), prompted the present study on the use of NSR for cancer immunotherapy.

Initially, we investigated whether NSR-infected human monocyte-derived DCs (MoDCs) can activate $CD8^+$ T cells *in vitro*. MoDCs were derived from peripheral blood mononuclear cells of healthy donors by Ficoll-Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and cultured as previously described (14). Infection of DCs was remarkably efficient, resulting in over 90% green fluorescent protein (GFP)-positive cells under optimal conditions (data not shown). To study the T-cell activation capacity of infected DCs, an NSR variant was constructed that encodes the immunodominant NLVPMVATV epitope of human cytomegalovirus pp65 ($pp65_{495\text{-}503}$) fused to the C terminus of GFP (Fig. 1A). NSR particles encoding this fusion protein (NSR_{NLV}) and particles encoding GFP only (NSR_{GFP}) were used to infect DCs obtained from an HLA A2-positive donor. As a positive control, DCs were incubated with a synthetic NLVPMVATV peptide (1 μM), and culture medium was used as a negative control. After overnight incubation, HLA A*0201-restricted, NLVPMVATV-specific $CD8^+$ T cells (14) were added to the DCs and cocultured for 4.5 h. T cells were harvested, stained for CD3, CD8, and CD107a (LAMP-1) surface markers, and subsequently intracellularly stained for IFN-γ and tumor necrosis factor (TNF). The results of this experiment demonstrate that NSR can be successfully used as a vector to deliver specific immunogenic epitopes to human DCs, which trigger an effector function in corresponding $CD8^+$ T cells (Fig. 1B).

To investigate the feasibility of using NSR for cancer immunotherapy, we made use of C57BL/6 Kh (B6, H-2b) mice and E.G7-OVA cells, a chicken ovalbumin (OVA) gene-transfected clone of mouse lymphoma EL4 cells obtained from the American Type Culture Collection (Manassas, VA, USA). (The animal experiments described here were conducted in accordance with the

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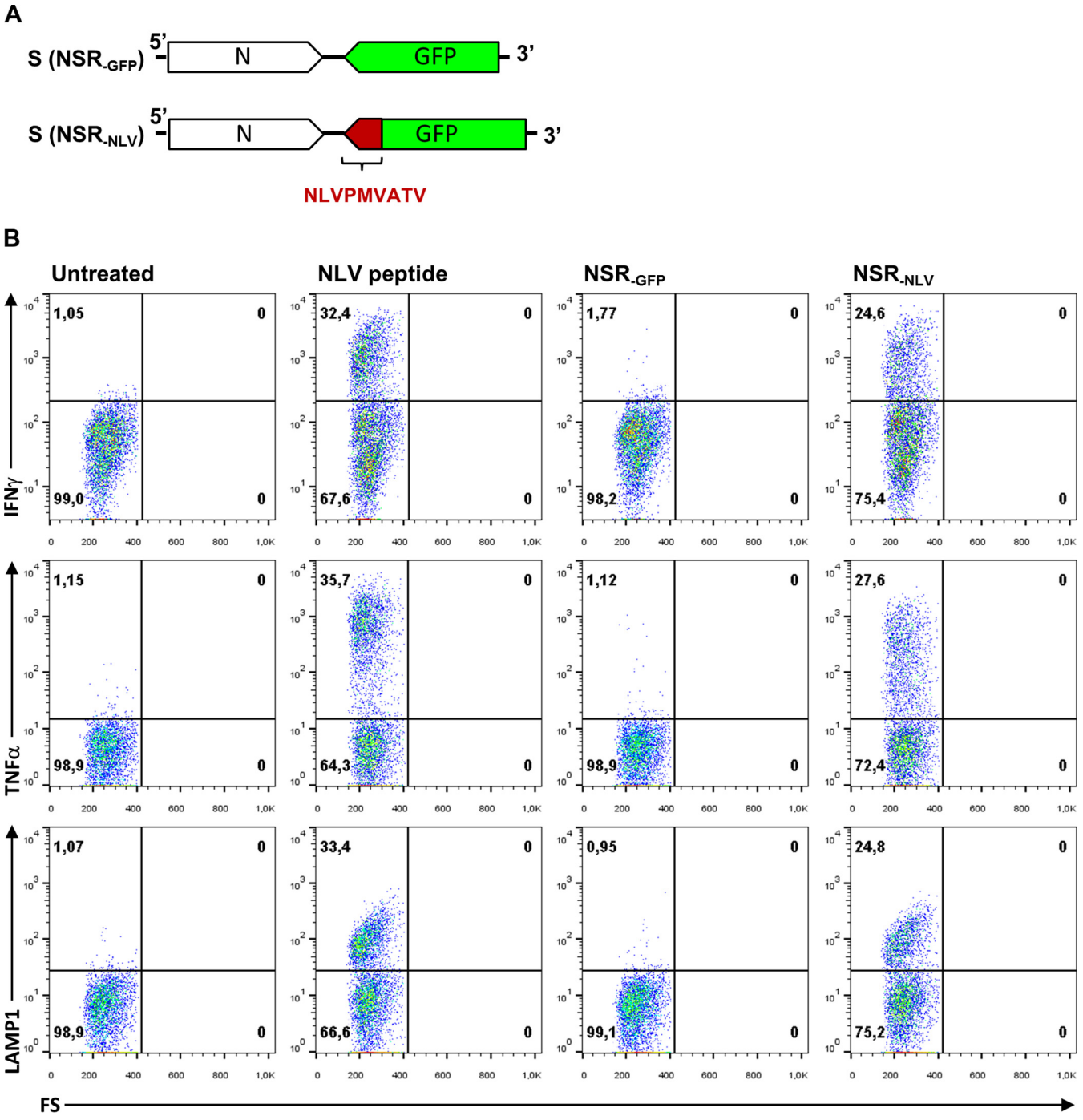

FIG 1 (A) Schematic representation of the NSR S segment encoding GFP (top) or the C-terminally fused $pp65_{495\text{-}503}$ epitope (bottom) in an antigenomic orientation. (B) NSR-infected human DCs activate antigen-specific $CD8^+$ T cells. HLA $A2^+$ DCs were infected with NSR_{GFP} or NSR_{NLV} and cultured overnight. As a negative control, DCs were left untreated; as positive control, DCs were loaded with synthetic peptide $pp65_{495\text{-}503}$. An A2-restricted $CD8^+$ T cell clone specific for the $pp65_{495\text{-}503}$ epitope was added for 4.5 h of incubation in the presence of GolgiStop (Becton Dickinson) and analyzed for activation. The expression of IFN-γ, TNF, and LAMP-1 in the CD8/CD3-positive cell population is depicted. All three parameters are induced specifically in NSR_{NLV}-infected cells and not in NSR_{GFP}-infected cells. FS, forward scatter.

Dutch Law on Animal Experiments [Wod, ID no. BWBR0003081] and approved by the Animal Ethics Committee of the Central Veterinary Institute [permit no. 2014101.c].) An NSR variant was constructed that encodes the CD8-restricted SII NFEKL epitope of OVA ($OVA_{257\text{-}264}$) fused to GFP (NSR_{OVA}) (Fig. 2A). To determine whether NSR_{OVA} vaccination elicits a cellular immune response specific for this epitope, mice were vaccinated twice via the intramuscular route (thigh muscle), at a 1-week interval, with 10^8 50% tissue culture infective doses of NSR_{OVA} (Fig. 2B). Control mice were vaccinated with NSR_{GFP}.

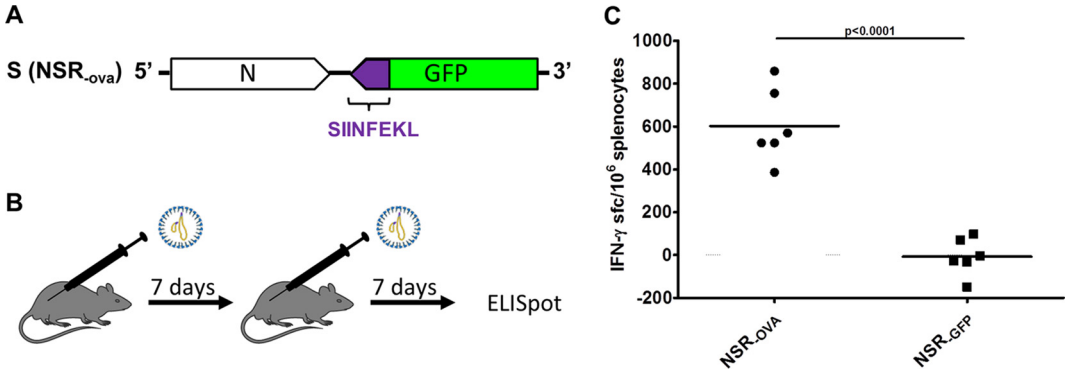

FIG 2 (A) Schematic representation of the NSR S segment encoding the $OVA_{257-264}$ peptide fused to GFP in an antigenomic orientation. (B) Representation of the regimens used for vaccination with NSR_{OVA} or control NSR_{GFP} vaccine. (C) ELISPOT assay showing IFN-γ responses of splenocytes collected from mice that were vaccinated with either NSR_{GFP} or NSR_{OVA} ($n = 6$). Symbols represent individual counts of IFN-γ spot-forming cells (sfc). Statistical significance (Student's *t* test) is indicated.

One week after the second vaccination, spleen cells were collected and evaluated for the ability to produce IFN-γ after stimulation with a synthetic SIINFEKL peptide (10 μg/ml; Invitrogen) in an enzyme-linked immunospot assay (ELISPOT) assay (10). This experiment demonstrated that NSR_{OVA} vaccination elicits a SIINFEKL-specific cellular immune response (Fig. 2C).

We finally asked whether prophylactic or therapeutic vaccination with NSR_{OVA} can reduce the outgrowth of E.G7-OVA tumor

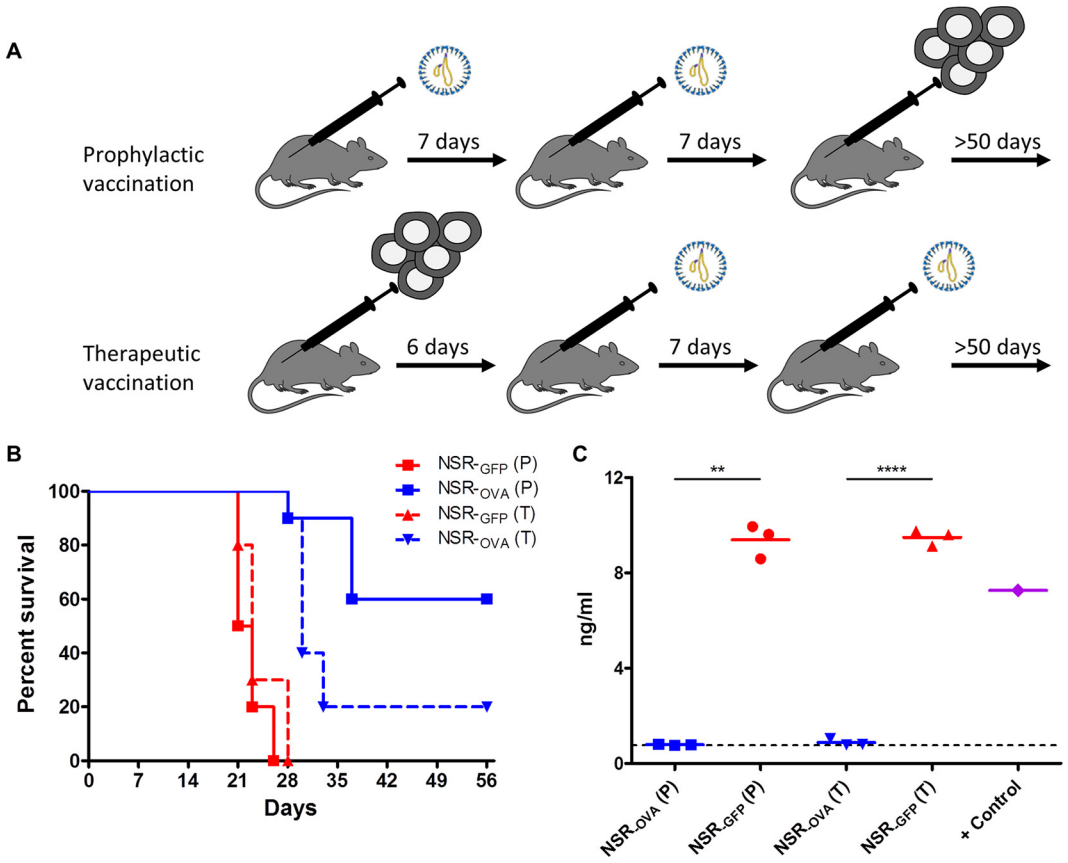

FIG 3 (A) Prophylactic and therapeutic regimens used for vaccination with NSR_{OVA} or control NSR_{GFP} vaccine. The primary therapeutic vaccination was applied when more than 50% of the E.G7-OVA-inoculated mice had palpable tumors. (B) Survival curves of mice prophylactically (P, solid lines) or therapeutically (T, interrupted lines) vaccinated with NSR_{GFP} or NSR_{OVA}. Statistical analyses were performed with GraphPad Prism. Log-rank (Mantel-Cox) tests revealed statistically significant differences ($P < 0.0001$) between NSR_{GFP} and NSR_{OVA} vaccinations with either a prophylactic or a therapeutic regimen. (C) Detection of OVA in supernatants of cultured E.G7-OVA cells by ELISA. Cells were recultured for 7 days after being collected from mice ($n = 3$/group) that had reached the humane endpoint. Statistical significance (Student's *t* test with Welch's correction for unequal variances) is shown. E.G7-OVA cells that were used for the inoculation of mice were used to obtain a positive-control (+ Control) sample.

cells (Fig. 3A). Mice were subcutaneously inoculated with 10^6 E.G7-OVA cells and euthanized by cervical dislocation when the tumor size reached 5,000 mm^3. Both prophylactic and therapeutic vaccinations via the intramuscular route with NSR_{OVA} resulted in increased survival times compared to those of control mice that had received NSR_{GFP} (Fig. 3B). Therapeutic vaccination resulted in complete tumor clearance in 2/10 mice, whereas prophylactic vaccination resulted in clearance of tumors in 6/10 mice. As expected, recultured tumor cells collected from NSR_{GFP}-vaccinated mice upon necropsy were found to express OVA, as determined by a commercial OVA enzyme-linked immunosorbent assay (ELISA; Agro-Bio). Surprisingly, tumor cells collected from mice vaccinated with NSR_{OVA} did not express detectable levels of OVA (Fig. 3C). This finding suggests that small numbers of the inoculated cells did not express OVA or lost OVA expression in time and that tumor cells expressing OVA were efficiently cleared by either prophylactic or therapeutic vaccination.

The present work demonstrates that NSR particles can successfully deliver an immunogenic peptide to human DCs and that these cells are capable of activating $CD8^+$ T cells. In addition, vaccination of mice with NSR expressing a single CD8-restricted epitope resulted in the complete clearance of lymphoma cells expressing the targeted antigen. Future experiments will address the efficacy of NSR vaccination to control outgrowth of tumors expressing self-antigens. Considering that self-antigens are much less immunogenic, NSR particles will be designed to express not only $CD8^+$ peptides but also $CD4^+$ and B-cell epitopes.

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