

Glycoprotein from street rabies virus BD06 induces early and robust immune responses when expressed from a non-replicative adenovirus recombinant

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Abstract The rabies virus (RABV) glycoprotein (G) is responsible for inducing neutralizing antibodies against rabies virus. Development of recombinant vaccines using the G genes from attenuated strains rather than street viruses is a regular practice. In contrast to this scenario, we generated three human adenovirus type 5 recombinants using the G genes from the vaccine strains SRV9 and Flury-LEP, and the street RABV strain BD06 (nrAd5-SRV9-G, nrAd5-Flury-LEP-G, and nrAd5-BD06-G). These recombinants were non-replicative, but could grow up to $\sim 10^8$ TCID₅₀/ml in helper HEK293AD cells. Expression of the G protein was verified by immunostaining, quantitative PCR and cytometry. Animal experiments revealed that immunization with nrAd5-BD06-G can induce a higher seroconversion rate, a higher neutralizing antibody level, and a longer survival time after rabies virus challenge in mice when compared with the other two recombinants. Moreover, the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) was significantly higher in mice immunized with nrAd5-BD06-G, which might also contribute to the increased protection.

These results show that the use of street RABV G for non-replicative systems may be an alternative for developing effective recombinant rabies vaccines.

Introduction

Rabies is a neglected zoonosis that causes more than 55,000 annual human deaths worldwide, mostly in Asia and Africa, where dogs are the major reservoir responsible for transmission to humans in the region [35]. Mass dog vaccination has proved to be the most cost-effective measure for rabies control [9, 18]. Vaccine development for dogs is still the focus of applied research, especially the development of vaccines for free-ranging dogs. Oral vaccination is one milestone for the prevention and control of wildlife rabies in North America, Europe, Israel, and elsewhere [22, 36]. Currently, two recombinant vaccines are being used for oral rabies vaccination in wildlife. The first recombinant vaccine, V-RG, developed more than two decades ago, is based on vaccinia virus expressing the glycoprotein (G) from the rabies virus (RABV) vaccine strain ERA [3, 15, 37]. V-RG was licensed conditionally in 1995 in the USA for oral vaccination of wildlife. The success of V-RG in rabies control comes with limitations, such as inducing insufficient virus neutralizing antibody (VNA) responses in skunks or dogs by single-dose vaccination [4, 11, 27]. Moreover, human vaccinia virus infections have been associated with contact with V-RG vaccine baits [5]. The second recombinant vaccine, AdRG1.03 (trade name ONRAB[®]) based on human adenovirus type 5, has been approved for field trials in Canada since 2006 and is being tested in the USA [16, 17]. AdRG1.03 is replication-competent and can be re-isolated from excretions from

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vaccinated animals. The possible public-health impact of releasing this live virus into the environment is at an early stage of evaluation [16, 17, 22]. Of note, both V-RG and AdRG1.03 recombinants were constructed using the G gene from the vaccine strain ERA. Similar problems were also observed with the previous construct, CAV-E3Δ-CGS, which was based on replication-competent canine adenovirus type 2 expressing the G gene from vaccine strain SRV9 [12].

With the above concerns, development of a recombinant vaccine for oral immunization with excellent safety and high efficacy remains an important issue for rabies prevention and control. Efforts mainly focus on screening various virus vectors, such as vaccinia virus, adenovirus, herpesvirus and paramyxovirus vectors [1, 20, 21, 38].

Studies have shown that attenuated RABVs appeared to activate, while pathogenic RABVs evaded, the host innate immune responses, and the evasion was due to the restricted expression of the G protein [28, 33]. In street RABV strains, G protein expression is more controlled than in attenuated strains [24, 37]. Hence, recombinant vaccines using the G gene from street RABV have not been considered or tested in detail. However, the immune efficacy was due not only to the expression level of the G protein but also to the immunogenicity itself. Evaluation of the immune potential could be simplified in an isolated system, such as non-RABV recombinants, since the G protein in its own RABV system interacts with other viral components.

BD06 is a street RABV strain that was isolated from a rabid dog in the Baoding district of Hebei province, China, in 2006. Phylogenetically, BD06 belongs to the China clade 1, whose members are responsible for most rabies cases in humans and dogs in China [39]. Therefore, the development of a vaccine based on this virus could be an effective choice for rabies control in China. The non-replicative human adenovirus type 5 is a widely used virus vector with advantages of significant safety, advanced delivery efficiency, and ease of cultivation [2]. Here, using this delivery system, we demonstrate an alternative strategy for recombinant rabies vaccine development using a street RABV G gene.

Materials and methods

Plasmids, viruses and cells

The non-replicative RAPAd[®] CMV Adenoviral Expression System was purchased from Cell Biolabs Inc. The kit comprises an intermediate pacAd5 CMVK-NpA plasmid and a human adenovirus type 5 (HAdV5) backbone plasmid, pacAd5 9.2-100. The street RABV BD06 strain EU549783.1 was propagated and maintained in mouse

brains. The attenuated rabies vaccine strain Flury-LEP (GU565703.1) has been maintained routinely in the Veterinary Research Institute since 1996 after being introduced from the Culture Collection Center, China Veterinary Drug Control Institute (<http://www.ivdc.gov.cn>). The attenuated RABV strain, SRV9 (AF499686.2), was adapted and grown in BHK-21 cells. The challenge virus standard CVS-11 was cultured as described previously, and the CVS-24 strain was propagated and maintained in mice by intracerebral (i.c.) inoculation. Homogenized brain tissues were used for in vivo challenges [6]. The BHK-21 and human embryonic kidney cells (HEK293AD) (Cell Biolabs Inc.) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal bovine serum (Gibco) and penicillin-streptomycin at 37 °C with 5 % CO₂.

RABV G genes

The G genes of RABV BD06 and Flury-LEP were amplified from infected mouse brain tissues by reverse transcription polymerase chain reaction (RT-PCR) according to a previously described protocol [25]. The G gene of SRV9 was amplified from infected BHK-21 cells. The primers used for RT-PCR are listed in Table 1.

Generation of non-replicative recombinant adenoviruses

The G genes of SRV9, Flury-LEP, and BD06 were cloned individually into the intermediate plasmid pacAd5CMVK-NpA, resulting in the recombinant plasmids pacAd-SRV9-G, pacAd-Flury-LEP-G, and pacAd-BD06-G, respectively. Each recombinant plasmid, together with the backbone pacAd5 9.2-100 plasmid, was linearized by treatment with *Pac* I and used to co-transfect HEK293AD cells in a 6-well plate, using FuGENE[®] HD transfection reagent according to the manufacturer's instructions (Promega). The transfected cells were incubated at 37 °C in 5 % CO₂ for ~10 days until virus plaques became visible by light microscopy. The recovery of recombinant viruses was confirmed by electron microscopy. The recovered viruses were named nrAd5-SRV9-G, nrAd5-Flury-LEP-G, and nrAd5-BD06-G, respectively. A wild-type adenovirus virus without an insertion (wt-nrAd5) was also generated using the same procedure as a control. All recovered viruses were passaged 4 to 9 times in HEK293AD cells to enrich virus titers.

RABV G expression of the non-replicative recombinant adenoviruses in infected cells

To compare G protein expression among the three recombinants in non-supportive cells, BHK-21 cells were

Table 1 Primers designed for the present study

| Primer | Sequence 5' to 3' | Target gene (position) | Assay |
|---------------------------------|---|---|--------|
| SRV9-Gf | CCG <i>GAATC</i> CCACCATGGTTCCTCAGGCTCTCC | SRV9 G (nt1-19) ^a | RT-PCR |
| SRV9-Gr | CGC <i>GGATCC</i> TTACAGTCTGGTCTCACCC | SRV9 G (nt1575-1557) | RT-PCR |
| LEP-Flury-Gf | CCG <i>GAATC</i> CCACCATGGTTCCTCAGGTTCTT | Flury-LEP G (nt1-18) | RT-PCR |
| LEP-Flury-Gr | CGC <i>GGATCC</i> TCACAGTCTGGTCTCGCCTC | Flury-LEP G (nt1575-1556) | RT-PCR |
| BD06-Gf | CCG <i>GAATC</i> CCACCATGATTCTCAAGTTCTT | BD06 G (nt1-18) | RT-PCR |
| BD06-Gr | CGC <i>GGATCC</i> TCACAGCTTGGTCTCACTTC | BD06 G (nt1575-1556) | RT-PCR |
| Human actin β (sense) | ACCTTCTACAATGAGCTGCGTGTG | Human β -actin (nt349-372) ^b | qPCR |
| Human actin β (antisense) | CCGGAGTCCATCACGATGC | Human β -actin (nt551-533) ^b | qPCR |
| RABV-G (sense) | GTTCTAGGACTTAGACTTATGGA | SRV9 G (nt965-943) ^c | qPCR |
| RABV-G (antisense) | TGACTGAGACGTCTGAAACTCAC | SRV9 G (nt745-767) ^c | qPCR |

Restriction sites are indicated in *italic*. The sequences used for gene cloning are underlined

^a nt, nucleotide

^b Reference to NM_001101

^c Conserved among the three G genes

grown overnight on glass coverslips and infected individually with the recombinant virus nrAd5-SRV9-G, nrAd5-Flury-LEP-G, or nrAd5-BD06-G at a multiplicity of infection (MOI) of 0.1. At 48 hours postinfection, the expression of G protein was detected by direct fluorescent antibody testing (DFA) as described previously [7]. Briefly, the cells were fixed with 80 % acetone at 4 °C for 20 min and stained with FITC-conjugated mouse anti-G (1B12) monoclonal antibodies (Veterinary Research Institute, Changchun, China). After washing with PBS (0.01 M, pH 7.4), the results were recorded using a laser scanning confocal microscope (Olympus FluoViewTM FV1000, Olympus Corporation).

For quantitative RT-PCR (RT-qPCR), HEK293AD cells were infected with recombinant adenoviruses at an MOI of 0.1. After incubation at 37 °C for 48 hours, the total RNA of infected HEK293AD cells was extracted using an RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions and treated with RNase-free DNase I to remove contaminating genomic DNA. cDNAs were produced using PrimeScriptTM RT Master Mix (Perfect Real Time) (Takara), following the supplier's instructions. qPCR assays targeting the G genes were performed in an Mx3000P multiplex quantitative PCR system (Stratagene) using SYBR[®] Premix Dimer EraserTM (Perfect Real Time) (Takara) according to the manufacturer's instructions. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [29]. Expression of β -actin was used as an internal control, and cDNA of HEK293AD cells infected with nrAd5-BD06-G was used as a reference sample. The qPCR was performed in triplicate and repeated at least three times independently. Primers used for qPCR are shown in Table 1.

For flow cytometry, infected cells were harvested by trypsin digestion 48 hours after infection. The cells were collected and resuspended in 2 ml of 0.01 M PBS (pH 7.4). After fixation with 80 % acetone at 4 °C for 30 min, the cells were collected again by centrifugation and were stained with FITC-labeled mouse anti-G (1B12) monoclonal antibodies at 37 °C for 1 hour. The stained cells were counted using a BD FACSCalibur Flow Cytometer (BD biosciences) [31].

Safety evaluation of the recombinant adenoviruses in mice

All animal tests were performed in accordance with Animal Welfare Guidelines of the Academy of Military Medical Sciences (Changchun, China), and all efforts were made to minimize suffering. To test possible virulence of the recombinants, we delivered the viruses to mice by different administration routes (oral, i.c. and intramuscular [i.m.]). Ninety female Kunming mice (13-16 g, Institute of Changchun Biological Products, Changchun, China) were randomly assigned to nine groups, with 10 mice in each group. The titer of three recombinant viruses was adjusted to $10^{7.0}$ TCID₅₀/mL before inoculation. The mice were received 100 μ l orally, 30 μ l i.c. and 50 μ l i.m. for each recombinant. The animals were observed daily for any adverse reactions for 28 days. During the observation period, in the oral administration group, feces from each mouse were collected for possible isolation of excreted virus. The feces were soaked in 0.01 M PBS (pH 7.4) and the supernatants were filtered and cultured in HEK293AD cells. Any cytopathic effect (CPE) of the treated cells was monitored for 96 hours.

Immune responses in mice after inoculation with the recombinant adenoviruses

To analyze innate immune responses after i.m. administration, we randomly divided 100 female Kunming mice (13–16 g, Changchun Institute of Biological Products, Changchun, China) into five groups, including nrAd5-BD06-G, nrAd5-SRV9-G, nrAd5-Flury-LEP-G, wt-nrAd5 as a negative control, and HEK293AD cell lysates as a blank control. Each mouse was inoculated with 50 μ l of virus at a concentration of 10^7 TCID₅₀/ml or 50 μ l of HEK293AD cell lysate. Blood was sampled from the retro-orbital plexus at 0, 24, 48, 72 and 96 h post-injection. Innate-immune-response-related cytokines, including IL-1 β , GM-CSF, MCP-1 and SCF, were measured using an ELISA kit according to the manufacturer's instructions (Signosis).

Similar to the protocol for detecting innate immune responses, blood samples were collected from each group of mice every 2 weeks after immunization, and the sera were tested for virus neutralizing antibody (VNA) by the fluorescent antibody virus neutralization (FAVN) test [6]. Briefly, 3-fold serial dilutions of standard serum (0.5 IU/ml) and test serum samples were prepared in quadruplicate in a multi-well plate, and mixed with 50 μ l of CVS-11. After incubation at 37 °C in a humidified 5 % CO₂ incubator for 1 hour, a 50- μ l suspension containing 2×10^4 BHK-21 cells was added, and the incubation was continued for 48 hours. The cells were fixed at 4 °C by treatment with 80 % acetone for 30 min and stained with FITC-labeled mouse anti-RABV-N monoclonal antibodies (Veterinary Research Institute, Changchun, China). Fluorescence was observed by UV microscopy (Olympus), and the VNA titers were calculated using the Spearman-Kärber formula.

Animal survival rate challenge

In parallel to the tests of innate and humoral immune responses, similar groups of mice were compared in a challenge test. Twenty-one days after immunization by i.m. injection using 50 μ l of each recombinant virus at a concentration of 10^7 TCID₅₀/ml, mice were challenged i.m. with 30 μ l of mouse brain suspension containing 120 LD₅₀ of CVS-24. The mice were observed for 21 days after challenge and were euthanized by CO₂ intoxication once any sickness was observed. The animal brain tissues were collected for confirmatory diagnosis using the DFA test.

Statistical analysis

Statistical significance was determined using Student's *t*-test. *P*-values less than 0.05 were considered statistically significant. All statistical analyses and graphing were performed using the software GraphPad Prism, version 5.01.

Results

Generation of non-replicative recombinant human adenoviruses

RABV G genes (SRV9-G, Flury-LEP-G, and BD06-G) from virus-infected cells were successfully amplified by RT-PCR and cloned into intermediate plasmid pacAdCMVK-NpA, yielding pacAd5-RABVG plasmids (pacAd5-SRV9-G, pacAd5-Flury-LEP-G, and pacAd5-BD06-G). The pacAd5-RABVG plasmid, together with human adenovirus type 5 backbone plasmid pacAd5 9.2-100, was linearized by treatment with *Pac* I and used to cotransfect HEK293AD cells. CPE became visible after incubation for about 7 days [2], and typical adenovirus particles were observed by electron microscopy after cell culture supernatants were concentrated by ultracentrifugation (data not shown). The growth dynamics of the recovered recombinant viruses nrAd5-SRV9-G, nrAd5-Flury-LEP-G, and nrAd5-BD06-G were compared with that of wt-nrAd5 in supplementary HEK293AD cells. Comparable virus growth was observed, and the titer of recombinant viruses reached over 10^8 TCID₅₀/ml, with wt-nrAd5 less than a half log higher (Fig. 1), indicating that the virus proliferation was only slightly affected by the expression of exogenous gene, if at all.

RABV G expression of the non-replicative recombinant adenoviruses in infected cells

G protein expression was detected in all recombinant-virus-infected non-supportive BHK-21 cells, but a weaker staining pattern was observed in nrAd5-BD06-G-infected cells than in nrAd5-SRV9-G-, or nrAd5-Flury-LEP-G-infected cells. The spread of virus was restricted to single

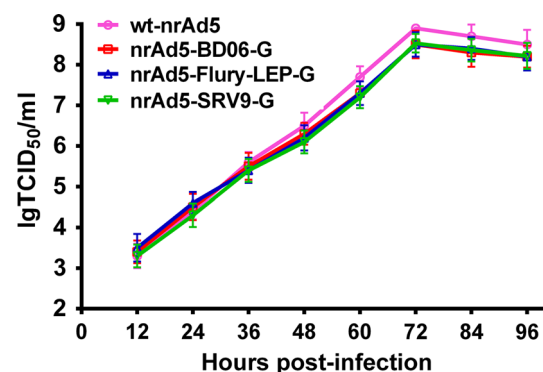


Fig. 1 Growth dynamics of recombinant viruses nrAd5-SRV9-G, nrAd5-Flury-LEP-G, and nrAd5-BD06-G. Virus titers in supernatants in HEK293AD cultures were tested at the indicated time points in triplicate. The virus growth curves were plotted using the virus titers. Data are shown as mean \pm standard error of the mean (SEM)

cells, indicative of the non-supportive nature of BHK-21 cells for replication of the recombinants (Fig. 2a).

The G mRNA transcripts in supportive HEK293AD cells were quantified by real-time RT-qPCR, as shown in Fig. 2b. The G mRNA amplifications followed the same trend. The relatively restricted expression of BD06 G mRNA was investigated.

Direct quantification of G proteins in supportive HEK293AD cells by flow cytometry, based on mean fluorescent strength, showed the same expression pattern as projected in real-time RT-qPCR. Relatively limited expression of BD06-G protein was also observed compared to the other constructs (Fig. 2c).

Safety evaluation of the recombinant adenoviruses in mice

No obvious adverse events were observed after administration of nrAd5-SRV9-G, nrAd5-Flury-LEP-G, or nrAd5-

BD06-G in mice, and all animals survived and were healthy at the end of the experiment. No RABV antigen was detected in brain tissues of euthanized mice, and no adenovirus was isolated from feces in the oral vaccination group.

Immune responses in i.m. vaccinated mice

Twenty-four hours after immunization, all recombinant viruses induced increased production of pro-inflammatory cytokines, including IL-1 β , MCP-1, SCF, and GM-CSF (Fig. 3). The concentrations of IL-1 β , MCP-1 and SCF induced by rAd5-BD06-G were higher statistically than the other two viruses and the controls. Specifically, the induction of GM-CSF was unique and dramatic after immunization with nrAd5-BD06-G.

The VNA titers and seroconversion rate in each animal group are shown in Fig. 4a and b. On day 14 post-vaccination, 85 % of the mice in the nrAd5-BD06-G group

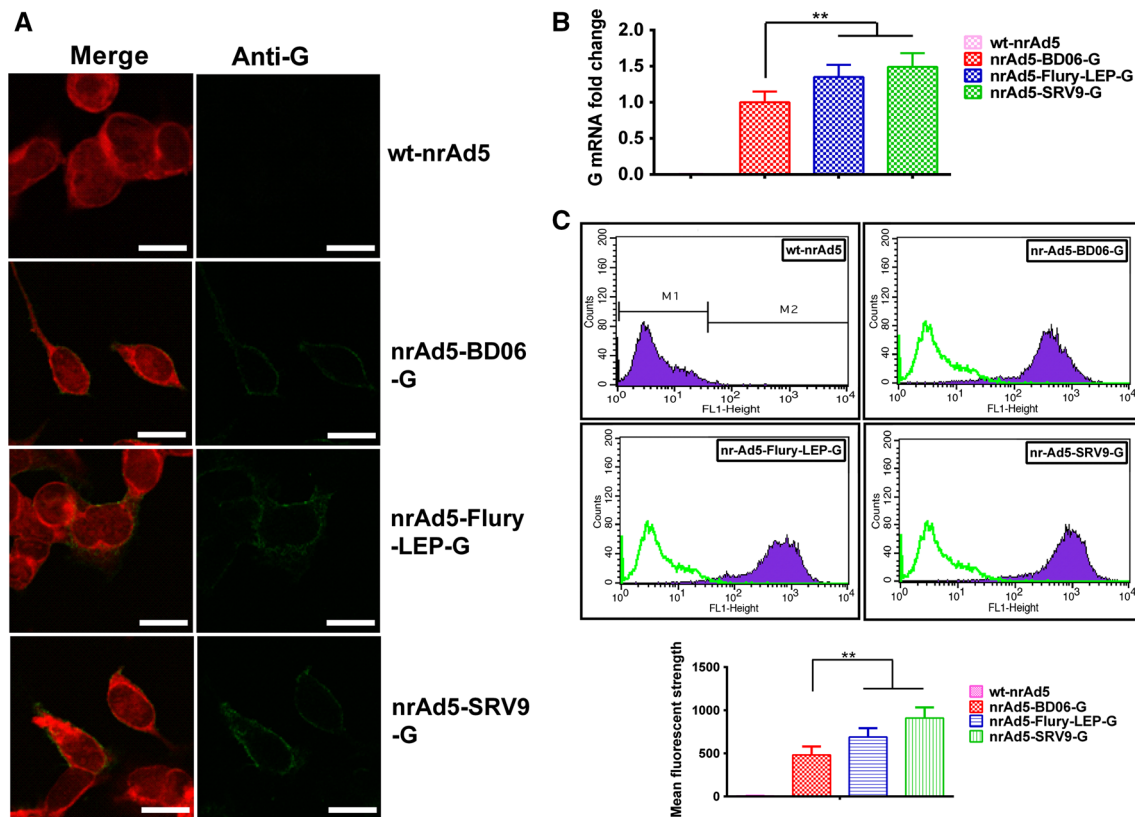


Fig. 2 Detection of the G protein expression from the recombinant viruses nrAd5-SRV9-G, nrAd5-Flury-LEP-G, and nrAd5-BD06-G. **a** Non-supportive BHK-21 cells were infected with each recombinant virus. G protein expression in the virus-infected culture was detected by confocal microscopy after fixation and staining with FITC-conjugated anti-G (1B12) and Evans blue (red staining). Bar, 10 μ m. **b** Total RNA was extracted from HEK293AD cells 48 h after infection with recombinant adenovirus. qPCR targeting the RABV G was performed in triplicate and relative expression levels of G protein

were calculated and normalized to that in nrAd5-BD06-G. Data are shown as mean \pm standard error of the mean (SEM). **, $p < 0.01$. **c** HEK293AD cells were infected with recombinant adenovirus nrAd5-SRV9-G, nrAd5-Flury-LEP-G or nrAd5-BD06-G. The infected cells were trypsinized and collected for quantitation by flow cytometry. The relative level of G protein expression was estimated from the mean fluorescent intensity. Data are shown as the mean of three independent assays \pm SEM. **, $p < 0.01$ (color figure online)

seroconverted (≥ 0.5 IU/ml), and the VNA titer was 17.24 ± 3.421 IU/ml (mean \pm standard deviation, SD); the seroconversion rate was increased to 95 % and the VNA titer reached 47.6 ± 10.2 IU/ml on day 28. In contrast, the groups nrAd5-SRV9-G and nrAd5-Flury-LEP-G exhibited restricted seroconversion rates and low VNA titers on day 14. Even though they increased on day 28, the seroconversion rates and VNA titers in animal serum samples vaccinated with nrAd5-SRV9-G and nrAd5-Flury-LEP-G were significantly lower than those of animals vaccinated with nrAd5-BD06-G.

Animal survival after challenge

As shown in Fig. 4c, 60 % of mice in the nrAd5-SRV9-G group survived CVS-24 challenge, 70 % in the nrAd5-Flury-LEP-G group, and 90 % in the nrAd5-BD06-G group. Twenty mice in each group were immunized i.m. and challenged i.m. 21 days post-immunization (dpi). The mouse survival rate was closely related to the seroconversion and VNA titers.

Discussion

One of the main challenges for global rabies prevention and control is effective delivery of rabies vaccines to the target animal species, especially to free-ranging dogs in the developing world. Rabies vaccines are very efficacious

when given by injection, but this route of administration is always unfeasible and expensive for vaccination of free-ranging animals. For example, fewer than 2 % of the dogs were vaccinated against rabies in some rural areas in China [12–14]. In addition to public-health awareness and education about animal-ownership responsibilities, which are complicated by social and economic status, development of an oral rabies vaccine for dogs would be significant in rabies control. The success of the V-RG recombinant vaccine for wildlife rabies control may not be easily adaptable to dog vaccination, due to the global eradication of and discontinued vaccination against smallpox, and potential safety concerns about V-RG distribution in developing countries, where close contact between animals and humans is frequent. An alternative strategy is to develop a replication-deficient recombinant that can be administered to target animals. Here, for proof-of-concept testing, we constructed three recombinants based on a replication-deficient human adenovirus type 5: nrAd5-SRV9-G, nrAd5-Flury-LEP-G, and nrAd5-BD06-G. In contrast to previous approaches, we found that nrAd5-BD06-G harboring a G gene from a street RABV strain induced an earlier and more robust immune response than the other two constructs with G genes from conventional vaccine strains. G protein expression from the three constructs in infected cells was observed by DFA, and measured using RT-qPCR and flow cytometry (Fig. 2). Restricted G protein expression, which has been observed previously for street RABVs, was also detected in our

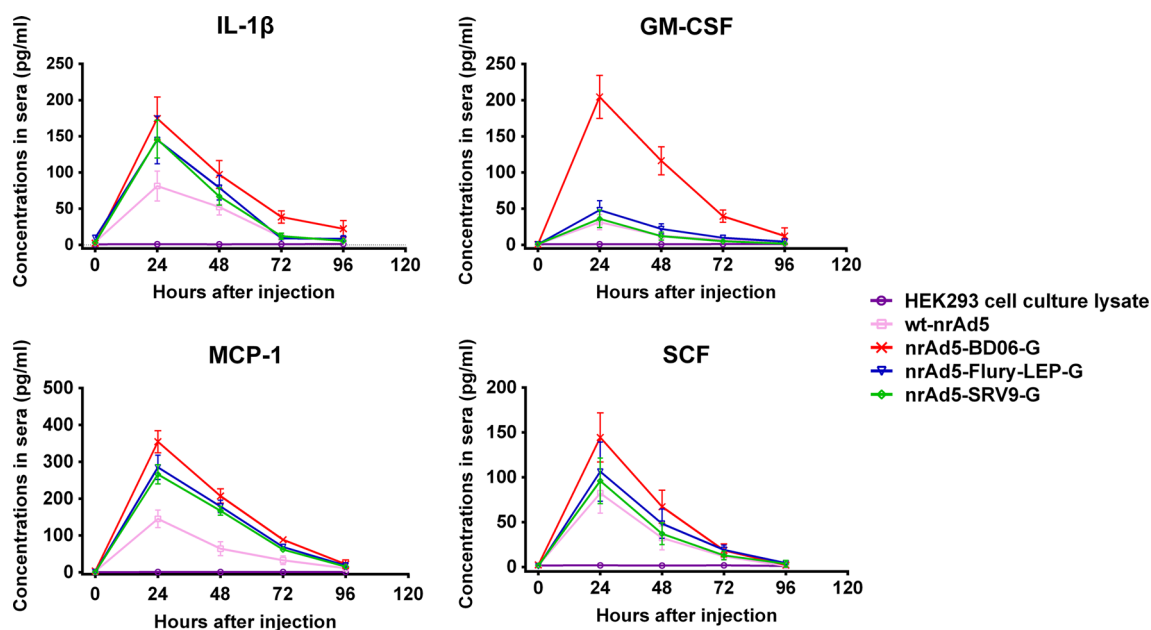
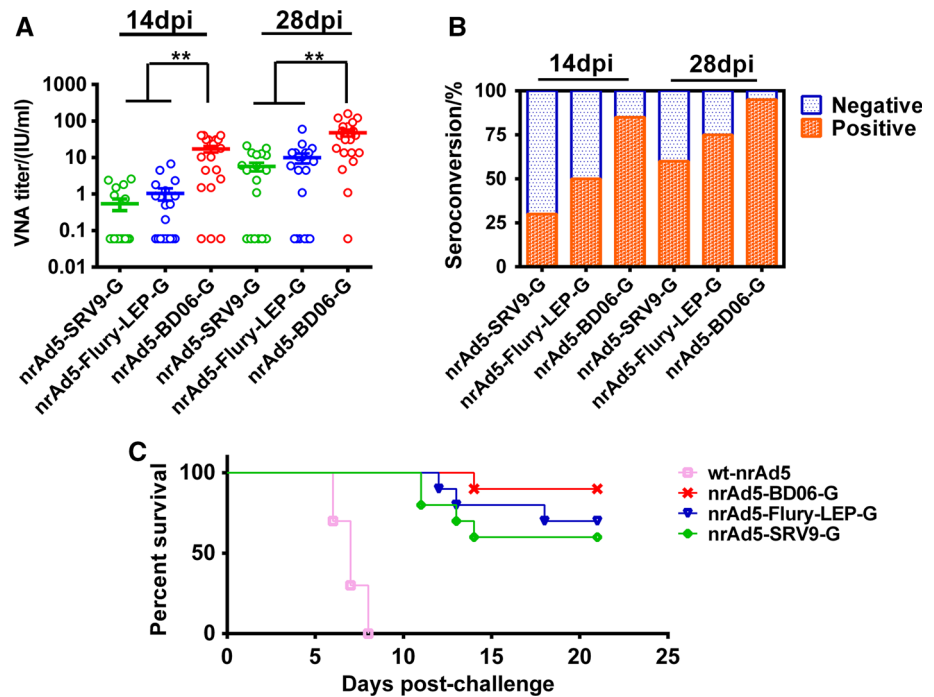


Fig. 3 Innate immune response induced by the recombinant viruses. Blood samples were collected at 0, 24, 48, 72 and 96 h from mice immunized with nrAd5-BD06-G, nrAd5-SRV9-G, nrAd5-Flury-LEP-G, or wt-nrAd5, or HEK293AD cell lysates as a blank control. Innate

immune response-related cytokines, including IL-1 β , GM-CSF, MCP-1 and SCF, were measured by ELISA. Data are shown as mean \pm SEM

Fig. 4 Immunogenicity of the recombinant viruses. Mice were immunized i.m. using 50 μ l of recombinant viruses at a concentration of 10^7 TCID₅₀/ml. **a** Blood samples were collected at 14 and 28 dpi. Titers of neutralizing antibody against RABV were determined by FAVN and are presented as an open circle. **, $p < 0.01$. **b** The seroconversion rates were calculated using the cutoff value of 0.5 IU/ml. **c** Immunized mice were challenged i.m. with 120 LD₅₀ of CVS-24 at 21 dpi and observed for another 21 days. Curves represent the survival rate vs. time



replication-deficient adenovirus system. However, the higher mouse survival rate did not correspond to the increased G protein level, but rather to early seroconversion and VNA titers (Fig. 4).

The reason for the restricted G protein expression in street-RABV-infected cells and tissues is still unknown [24, 37]. In our system, since the three G genes were in the context of the same vector, we do not know how the G protein was expressed differentially in infected cells. For example, Palusa et al. found that the G mRNA is differentially overexpressed, and the overexpression was mainly attributed to differences in the 3' untranslated region (UTR) of RABV G mRNA, which interacts with cellular PCBP2 protein and promotes transcription stability [26]. The 72-base 3' UTR is conserved in RABVs. In our recombinant viruses, the G gene coding sequences do not contain the 72-base 3' UTR, and gene expression is controlled by the same regulatory machinery. From the results shown in Fig. 2, we believe that G protein expression is unlikely to be controlled or restricted by its own coding sequence, but possibly through an unknown mechanism of virus-host cell interactions. Therefore, we infer that incorporation of the G gene coding sequence alone from street RABV into a replication-defective vector does not necessarily result in enhanced virulence or immune deficiency. There is no report that the immunogenicity of purified G protein from virulent/street RABVs is inferior to that of G protein extracted from attenuated RABVs. Furthermore, there is also no report that purified G protein from street RABVs causes neuronal damage after injection into animals,

although sequence similarity between RABV G protein and snake venom was found decades ago [19]. Although G expression was relatively low in cells, nrAd5-BD06-G induced earlier seroconversion, higher VNA, and a significantly increased survival rate in mice. The increased animal protection and early immune response may be due to the dramatic increase of GM-CSF, which has recently been linked to the regulation of CD8⁺ T cells and enhanced innate and adaptive immune responses [32, 34].

Using G genes from street RABVs for molecular engineering could potentially increase neuro-invasiveness of the recombinant virus, since RABV G is responsible for neurotropism. For example, lentivirus-vectored RABV G was engineered successfully to be neurotropic, and the pseudotyped virus was transported to the central nervous system (CNS) after peripheral injection [23]. In our investigation, because of the non-replicative characteristics of the recombinant viruses, enhanced neurotropism of nrAd5-BD06-G was not observed, and i.c. injection of nrAd5-BD06-G did not cause apparent damage to the CNS. Indeed, incorporation of a G gene from a street RABV into a replication-competent system could be a concern for neurotropism and possible neurotoxicity. In fact, all replication-competent attenuated RABVs, including ERA, still kill neonatal or suckling animals after peripheral and i.c. injection [8, 10, 30]. The encouraging discovery of nrAd5-BD06-G in our investigation is its safety profile and induction of a robust immune response after administration. The strategy of using G genes from street RABVs for non-replicative

systems may serve as an alternative in rabies vaccine development.

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