Supporting Information

Faber et al. 10.1073/pnas.0905640106

SI Text

Construction of Recombinant RV cDNA Clones. To facilitate insertion of a third GAS gene, *Asi*SI and *Asc*I restriction sites were introduced into pSPBNGAS-GAS. A fragment between *Pac*I and *Bsi*WI of pSPBNGAS, containing regulatory and intergenic sequences, was amplified using Deep Vent polymerase (New England Biolabs) and primers InterG *BA*(+) (5′-CGA TGT ATA CGT ACG TTT TT**G CGA TCG C**CG TCC TTT CAA CGA TCC AAG TC-3′[*Bsi*WI site underlined; *Asi*SI site in boldface]) and InterG *AN*(−) (5′-CTT AGC *GCT AGC* AAA AA**G GCG CGC C**GG AGG GGT GTT AGT TTT TTT CAT G-3′[*Nhe*I site underlined; *Asc*I site in boldface]). The PCR product was digested with *Bsi*WI and *Nhe*I and ligated into RV vaccine vector pSPBNGAS, previously digested with *Bsi*WI and *Nhe*I, resulting in pSP*BAAN*GAS. To insert a second copy of GAS gene, GAS cDNA was amplified with primers that contain the *Asc*I and *Nhe*I sites: SADB19 AscI(+) (5′-CGA ATT TAT *TGG CGC GCC* AAG **ATG** GTT CCT CAG GCT CTC CTG-3′ [*Asc*I site underlined; start codon in boldface]) and SADB19 NheI(−) (5′-CTT ATC AGC TA*G CTA GC*T AG**T TA**C AGT CTG GTC TCA CCC CCA-3′ [*Nhe*I site underlined; stop codon in boldface]), digested with *Asc*I and *Nhe*I, and ligated into pSPBAANGAS, previously digested with *Asc*I and *Nhe*I resulting in pSPBAANGAS-GAS (3). A third copy of GAS gene was introduced in a similar manner into pSPBAANGAS-GAS (3) using primers: SADB19 *Bsi*WI(+) (5′-CGA TGT ATA CGT ACG AAG **ATG** GTT CCT CAG GCT CTC CTG-3′[*Bsi*WI site underlined; start codon in boldface]) and SADB19 *Asi*SI(−) (5′-GAA TCT AGA GCG ATC GCC GT**T TA**C AGT CTG GTC TCA CCC CCA-3′ [*Asi*SI site underlined; stop codon in boldface]) resulting in pSPBAANGAS-GAS-GAS (Fig. 1). To confirm that any observations made with the triple GAS construct are due to an increased expression of G and not to the increased genome size of RV vector, we constructed pSPBAAN-GAS-GAS(−)-GAS(−) in which all ATG codons of the last 2 GAS genes, were scrambled (the modified gene was synthesized de novo by GenScript).

RNA Isolation, Reverse Transcription, and Real-Time PCR. NA cells grown in T25 tissue culture flasks were infected with RV at a MOI of 5. After incubation for 1 h at 37 °C, the inoculum was removed and cells were washed 3 times with PBS, replenished with 6 mL RPMI medium 1640 containing 2% FBS, and incubated at 37 °C. At 12, 24, or 48 h, cells were washed with PBS and RNA was extracted using the RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. Isolation of RNA from the brain tissue, real-time PCR, and statistical analysis has been described in ref. 1. The primers, probes, and artificial gene standards used to determine CD4 and CD19 mRNA levels have been detailed elsewhere (2).

FACS Analysis. Monolayers of NA cells grown in T25 culture flasks were infected at a MOI of 5 and incubated for 12, 24, and 48 h at 37 °C. Cells were washed 3 times with PBS and dissociated using a cell stripper (Mediatech). After centrifugation, cells were resuspended and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were washed twice with PBS, blocked with PBS containing 2% BSA and 10 mM glycine for 1 h, and incubated with rabbit anti-RV G antibody (1:2,000) followed by Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen; 1:500). Flow cytometry was performed on an Epics XL flow cytometer (Beckman Coulter). Results were analyzed using FlowJo version 7.2 software.

Determination of Virus-Neutralizing Antibody. Sera were heat inactivated at 65 °C for 30 min, and neutralizing activity was determined by the rapid fluorescent focus inhibition test, as described elsewhere (3). The neutralization titer, defined as the inverse of the highest serum dilution that neutralized 50% of the challenge virus, was normalized to international units using the World Health Organization anti-RV antibody standard. Geometric mean titers were calculated from individual titers in each group.

Virus Titration. To determine virus yields, monolayers of NA cells in 96-well plates were infected with serial 10-fold virus dilutions as described in ref. 4. At 48-h postinfection (p.i.), cells were fixed in 80% acetone and stained with FITC-labeled rabies virus N protein-specific antibody (Centocor). Foci were counted using a fluorescence microscope, and virus titers calculated in FFU. All titrations of virus stocks were determined in triplicate.

Single-Step Growth Curves. Confluent NA cell monolayers grown in T25 culture flasks were infected with RV at a MOI of 5. After incubation for 1 h at 37 °C, inocula were removed and cells were washed 3 times with PBS, cells were replenished with 6 mL RPMI medium 1640 (Mediatech) containing 2% FBS and incubated at 37 °C. After infection, 100 μL tissue culture supernatant was removed at the indicated time points, and virus was titrated in quadruplicate on NA cells. Differences were analyzed using two-way ANOVA with Bonferroni's multiple comparison test.

1. Faber M, et al. (2007) Dominance of a nonpathogenic glycoprotein gene over a pathogenic glycoprotein gene in rabies virus. *J Virol* 81:7041–7047.
2. Phares TW, Kean RB, Mikheeva T, Hooper DC (2006) Regional differences in blood-brain barrier permeability changes and inflammation in the apathogenic clearance of virus from the central nervous system. *J Immunol* 176:7666–7675.
3. Wiktor TJ, Macfarlan RI, Foggin CM, Koprowski H (1984) Antigenic analysis of rabies and Mokola virus from Zimbabwe using monoclonal antibodies. *Dev Biol Stand* 57:199–211.
4. Wiktor TJ, Doherty PC, Koprowski H (1977) In vitro evidence of cell-mediated immunity after exposure of mice to both live and inactivated rabies virus. *Proc Natl Acad Sci USA* 74:334–338.

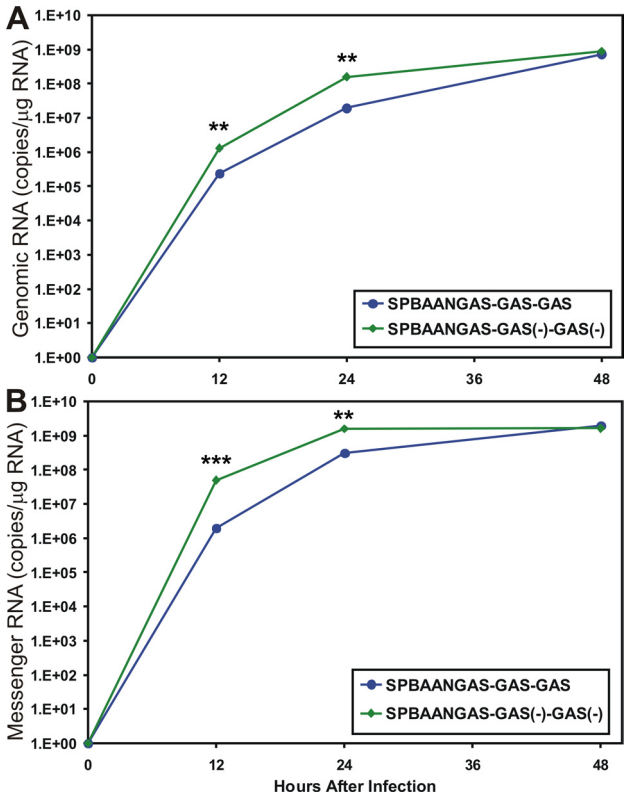

Fig. S1. Replication of viral genomic RNA (*A*) and transcription of viral mRNA (*B*) in NA cells infected with SPBAANGAS-GAS-GAS or SPBAANGAS-GAS(−)-GAS(−). Cells were infected at an MOI of 5, and total RNA was isolated at the indicated time points. A fragment of RV N mRNA or the N gene of genomic RV RNA was reverse transcribed and subjected to quantitative PCR analysis. The data were normalized using 18S rRNA as an internal control and analyzed using the comparative threshold cycle method. Data are means (±SE) of values for triplicate samples in 1 of 2 independent experiments with equivalent results. **, $P < 0.01$; ***, $P < 0.001$).

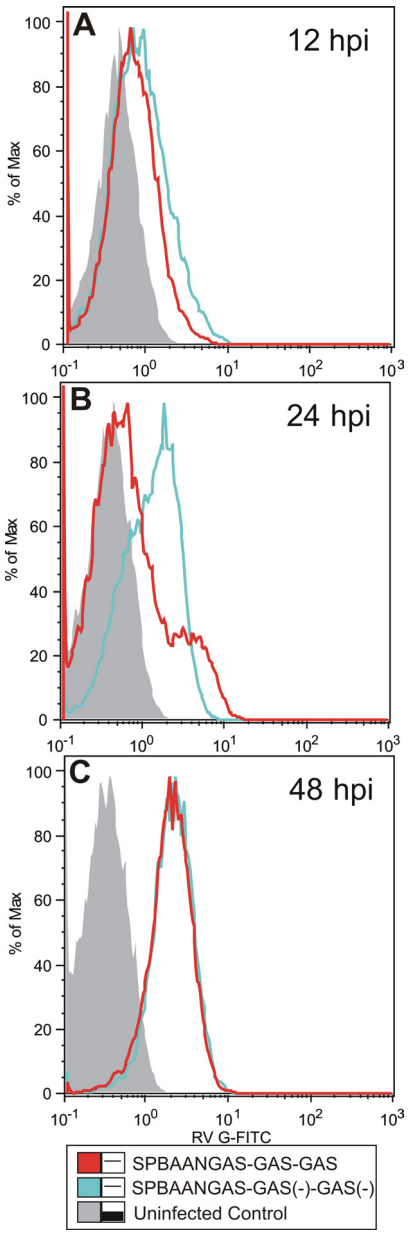

Fig. S2. RV G surface expression in NA cells infected with SPBAANGAS-GAS-GAS and SPBAANGAS-GAS(−)-GAS(−). NA cells were infected with SPBAANGAS-GAS-GAS or SPBAAN-GAS-GAS(−)-GAS(−) at MOI of 5. After incubation for 12 h (*A*), 24 h (*B*), or 48 h (*C*) at 37 °C, cells were stained with rabbit anti-RV G polyclonal antibody, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG. Samples were analyzed using an EPICS XL flow cytometer and FlowJo version 7.2 software. Data are representative of 3 independent experiments.