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## Supplemental Information

# Lyssavirus Vaccine with a Chimeric Glycoprotein Protects across Phylogroups

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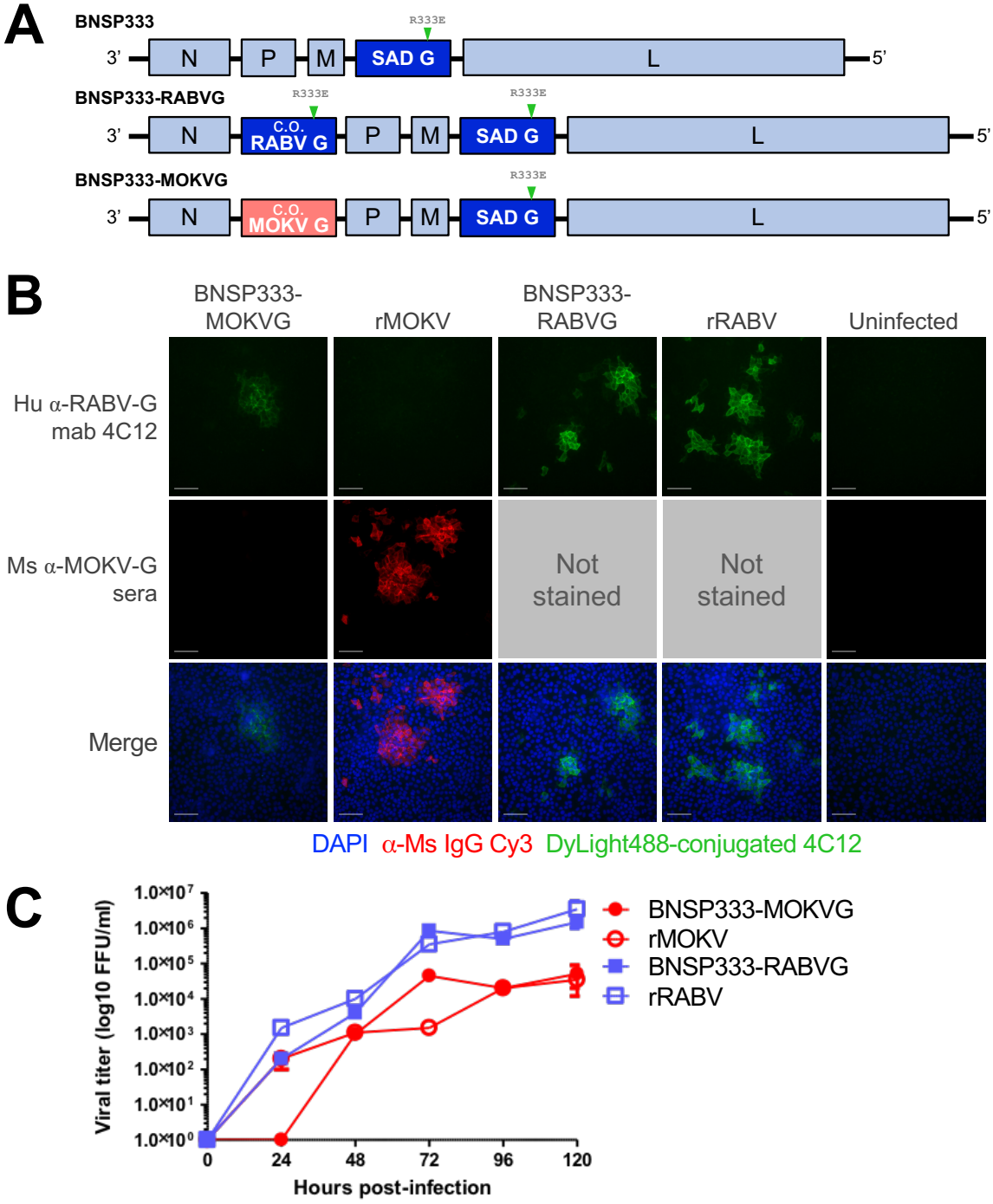


**Figure S1. Glycoprotein expression comparison in a dual G construct.** Related to STAR Methods. **(A)** Viral genome schematics. BNSP333 (top) is the parent vaccine vector based on RABV strain SAD B19. Its G is located in the native 4th position and contains the attenuating R333E mutation. BNSP333-RABVG contains an additional human codon-optimized RABV G at the 2nd position (also contains the attenuating R333E mutation); BNSP333-MOKVG contains an additional human codon-optimized MOKV G at the 2nd position. All RABV Gs in the above constructs have the attenuating R333E mutation. **(B)** Infection immunofluorescence. VERO cells infected with either BNSP333-MOKV-G (left column), rMOKV (second column), BNSP333-RABV-G (third column), rRABV (fourth column), or uninfected (right column) were fixed and stained with a DyLight 488-conjugated human anti-RABV G mAb 4C12 (green) and mouse anti-MOKV G sera (red). Nuclei labeled in blue by DAPI. Scale bars represent 100 μm. **(C)** Multi-step growth curve. BSR cells were infected at MOI 0.01.

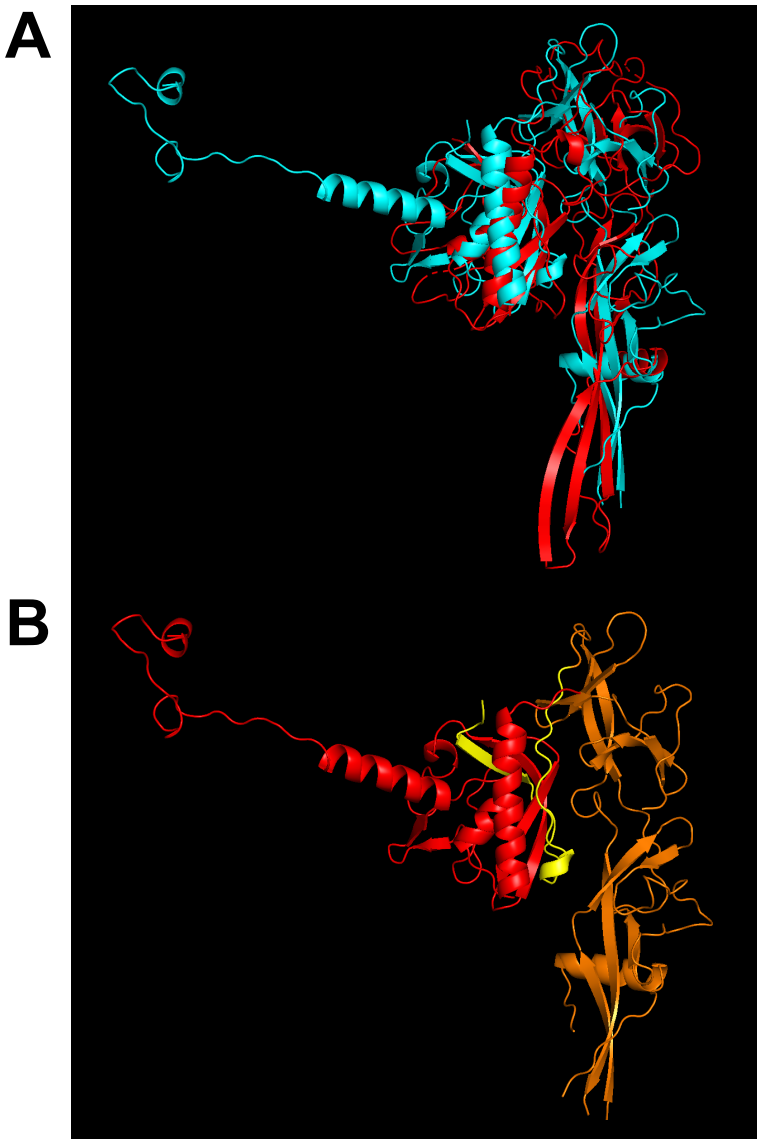


**Figure S2. Comparison between model and crystal structures of RABV G.** Related to Figure 1. **(A)** Overlay of structural model of RABV G generated using Phyre2 (red) and crystal structure of RABV G (cyan) (Yang et al., 2020). **(B)** Crystal structure of RABV G (Yang et al., 2020) colored to highlight the clip (yellow), core (orange), and flap (red) domains.

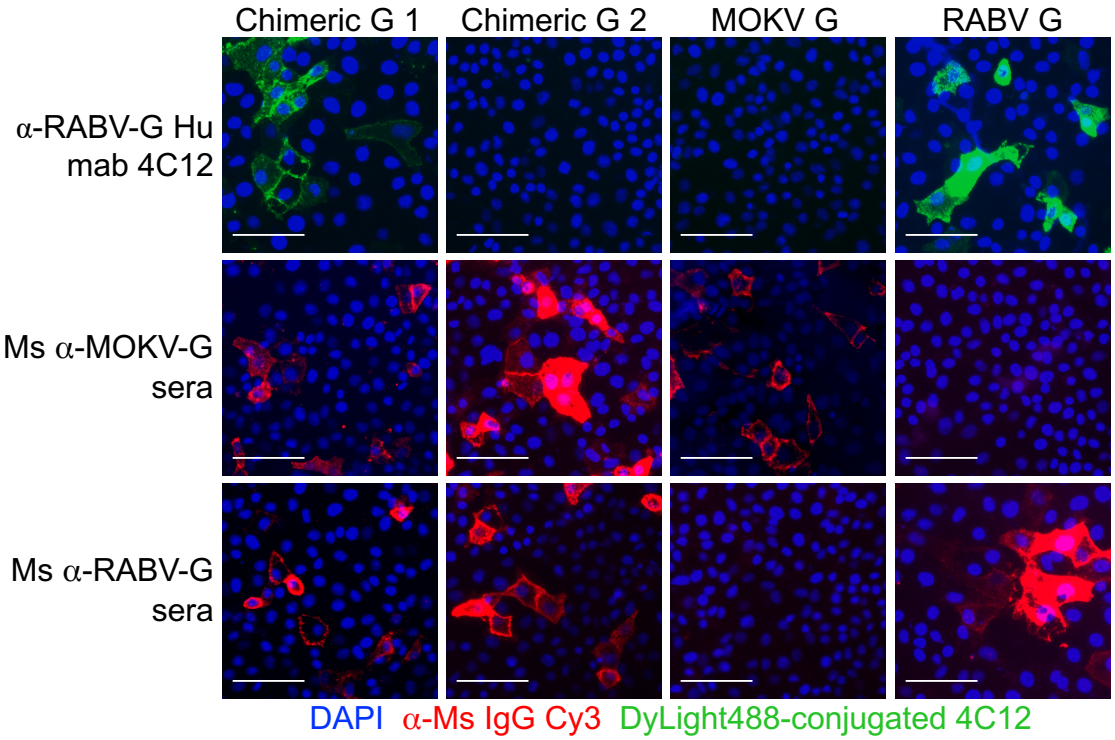


**Figure S3. Immunofluorescence of transfected chimeric lyssavirus glycoproteins.** Related to Figure 1. VERO cells transfected with pCAGGS expression plasmids containing the genes of either Chimeric G 1 (left column), Chimeric G 2 (second column), MOKV G (third column), or RABV G (fourth column). Two days post-transfection, cells were fixed with 4% paraformaldehyde and stained with a DyLight 488-conjugated human anti-RABV G mAb 4C12 (top row), mouse anti-MOKV G sera (middle row) or mouse anti-RABV G sera (bottom row). Sera specific to RABV G and MOKV G both bind to Chimeric Gs 1 and 2, whereas they are not otherwise cross-reactive with the other glycoprotein. 4C12 binds only to Chimeric G 1. Brightness adjusted with sets transfected with the same glycoprotein and stained with the same antibody. Scale bars represent 100 μm.

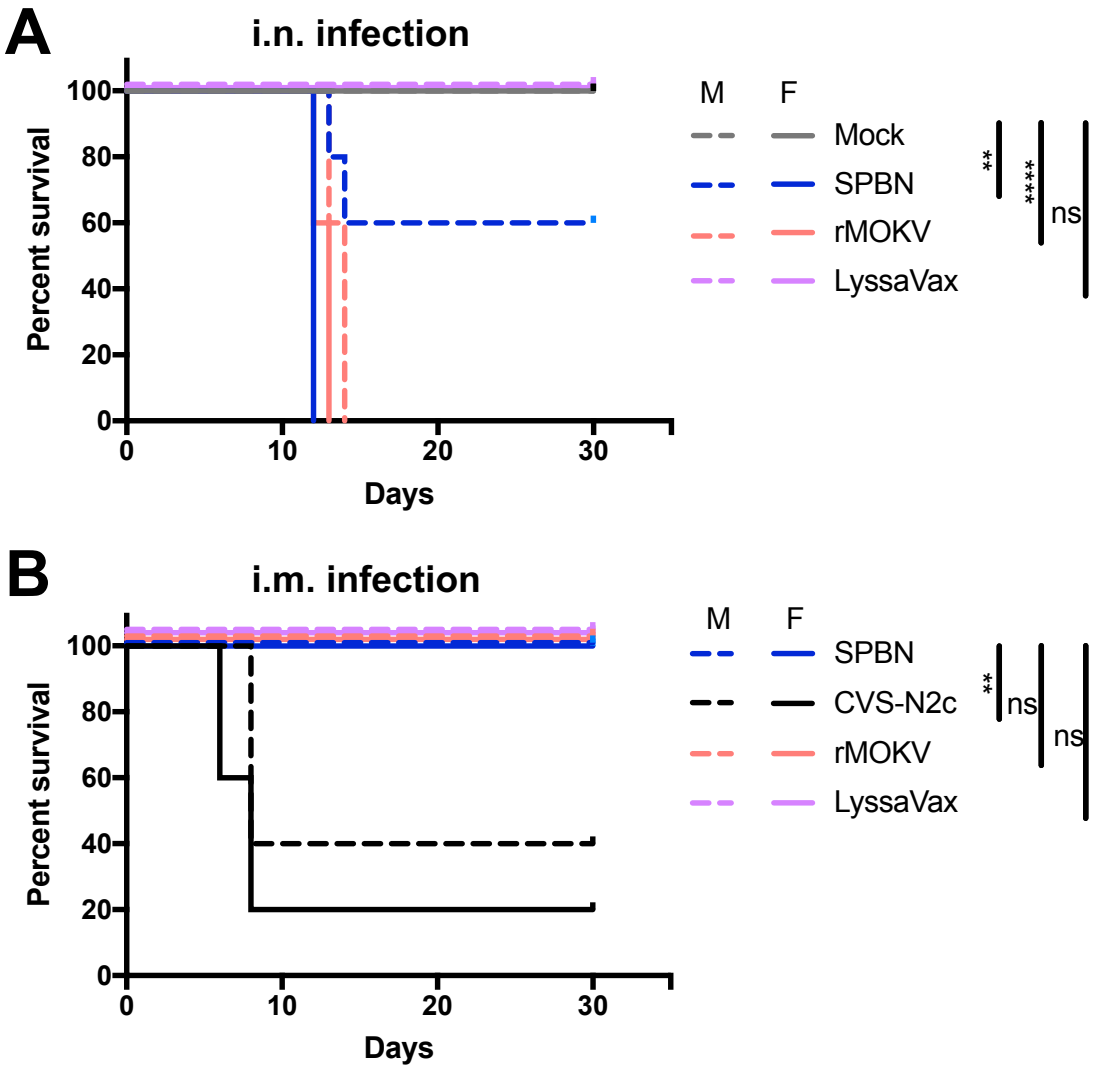


**Figure S4. Live virus pathogenicity profiles.** Related to STAR Methods. Survival after **(A)** intranasal or **(B)** intramuscular infection. Male (M, dashed line) and female (F, solid line) mice (n=5 per sex, per group) were inoculated with $10^5$ FFU of live virus and monitored for 28 days. Survival was analyzed using the log-rank Mantel-Cox test: **(A)** **, p=0.0011; ****p<0.0001, **(B)** **, p=0.0015. Statistical differences between male and female mice in **(A)**: SPBN, **, p=0.0027; rMOKV, *, p=0.0290; and **(B)**: CVS-N2c, ns.

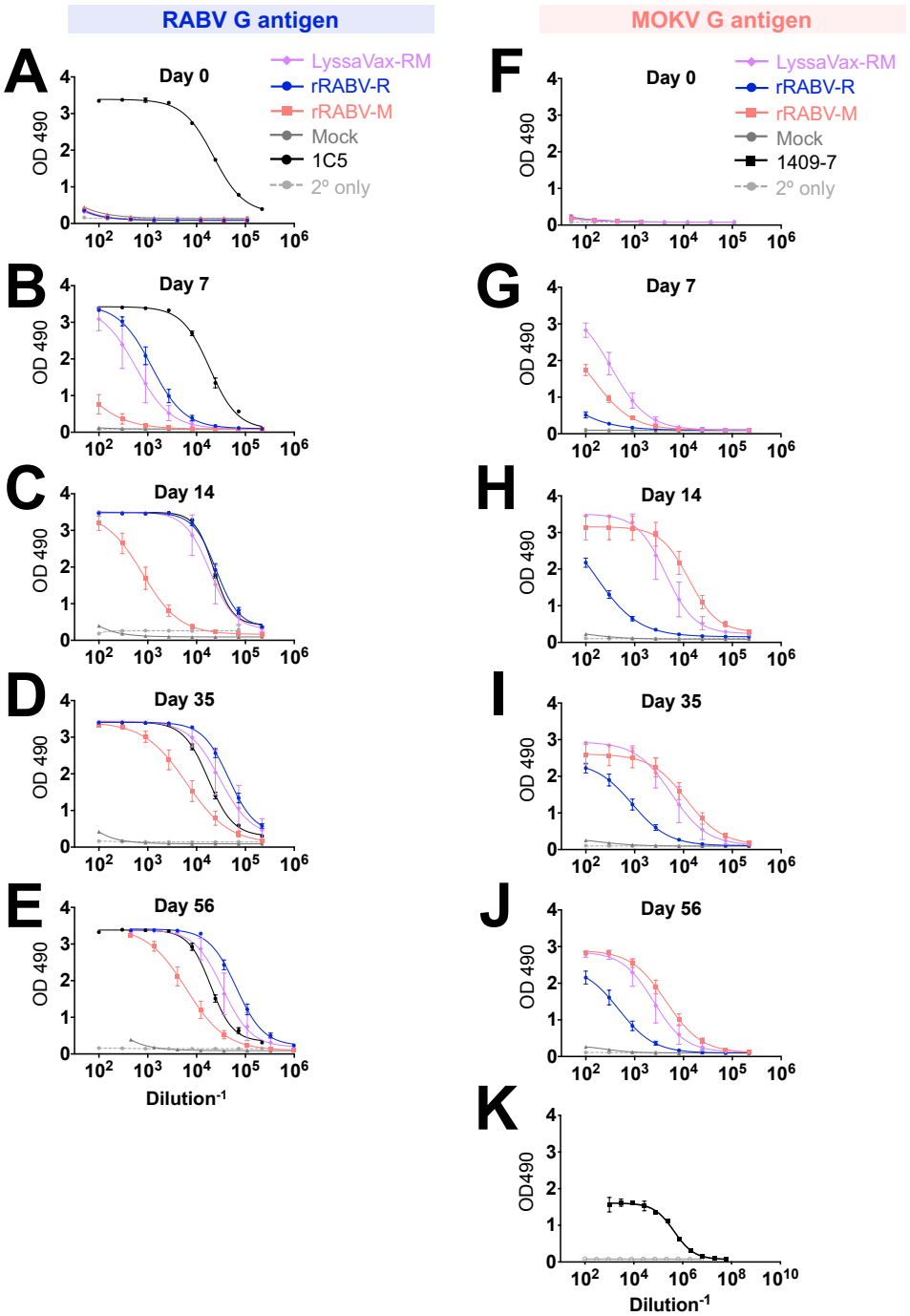


**Figure S5. Humoral response to recombinant LyssaVax (full dilution curves).** Related to Figure 3. Sera from mice immunized with rRABV (blue), rMOKV (red), LyssaVax (purple) or PBS (mock, dark gray) at days 0 **(A, F)**, 7 **(B, G)**, 14 **(C, H)**, 35 **(D, I)**, and 58 **(E, J)** post-immunization were assayed by ELISA against RABV G **(A-E)** or MOKV G **(F-J)** antigens (n=10 per group, mean ± SD). Control antibodies (black): 1C5 = mouse anti-RABV G mAb; 1409-7 = mouse anti-MOKV G mAb; 2º = goat anti-mouse IgG (H+L).

| | Mouse ID | Day 0 | 7 | 14 | 21 | 28 | 35 | 56 | Challenge | 96 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PBS | 1-1 | < 0.2 [a] | *No data* | < 4 [b] | < 4 [b] | < 0.2 [a] | < 4 [b] | < 0.2 [a] | SPBN | *Succumbed* | |
| | 1-2 | | | | | | | | | *Succumbed* | |
| | 1-3 | | | | | | | | | *Succumbed* | |
| | 1-4 | | | | | | | | | 10.5 | |
| | 1-5 | | | | | | | | rMOKV | *Succumbed* | |
| | 1-6 | | | | | | | | | *Succumbed* | |
| | 1-7 | | | | | | | | | *Succumbed* | |
| | 1-8 | | | | | | | | | *Succumbed* | |
| | 1-9 | | | | | | | | | *Succumbed* | |
| | 1-10 | | | | | | | | | *Succumbed* | |
| LyssaVax | 2-1 | < 0.2 [a] | < 4 | 13.8 | 9.9 | 26 | 19.1 | 33.8 | SPBN | 22.6 | |
| | 2-2 | | < 4 | 9.6 | 8.8 | 12.1 | 17.9 | 12.9 | | 10.3 | |
| | 2-3 | | < 4 | 5.2 | 6.1 | 11.1 | 9.6 | 6.1 | | 2 | *10.62* |
| | 2-4 | | < 4 | 19.0 | 22.4 | 21.4 | 26.2 | 20.4 | | 7.8 | *Avg. after SPBN challenge* |
| | 2-5 | | < 4 | 12.6 | 11.5 | 18.1 | 28.7 | 180 | | 10.4 | |
| | 2-6 | | < 4 | 8.0 | 6.2 | 9.5 | 23.3 | 20.9 | rMOKV | 2.7 | |
| | 2-7 | | < 4 | 6.0 | 9.9 | 21.9 | 76.7 | 166.8 | | 20.9 | |
| | 2-8 | | < 4 | 13.1 | 18 | 11.4 | 28.9 | 29.4 | | 2.7 | *6.46* |
| | 2-9 | | < 4 | 29.5 | 16.2 | 11.4 | 27.5 | 20.2 | | 2.7 | *Avg. after rRABV-M challenge* |
| | 2-10 | | < 4 | 37.7 | 60.4 | 86.2 | 76.2 | 81.3 | | 3.3 | |
| | *Avg.* | | | *15.45* | *16.94* | *22.91* | *33.41* | *57.18* | | *8.54* | |
| rRABV | 3-1 | < 0.2 [a] | < 4 | 24.5 | 17.7 | 25.6 | 34.9 | 9.9 | SPBN | 5.3 | |
| | 3-2 | | < 4 | 27.6 | 18.4 | 29 | 67 | 37.4 | | 137.8 | |
| | 3-3 | | < 4 | 12.0 | 6.2 | 9.1 | 28.4 | 34 | | 78.0 | *83.76* |
| | 3-4 | | < 4 | 8.1 | 5.3 | 18.2 | 85.1 | 7.5 | | 130.1 | |
| | 3-5 | | < 4 | 24.2 | 20.2 | 50.4 | 27.5 | 11.6 | | 67.6 | |
| | 3-6 | | 13.9 | 52.8 | 61 | 94.6 | 101.2 | 115.1 | rMOKV | 190.2 | |
| | 3-7 | | < 4 | 22.1 | 20.8 | 46.3 | 173.7 | 99.6 | | 196.4 | |
| | 3-8 | | < 4 | 23.8 | 18.3 | 34 | 60 | 16.8 | | 36.7 | *143* |
| | 3-9 | | 10.3 | 19.7 | 31 | 59.7 | 140 | 257.2 | | 138.0 | |
| | 3-10 | | 11.2 | 117.1 | 152.4 | 286.1 | 317.4 | 163.3 | | 153.8 | |
| | *Avg.* | | | *33.19* | *35.13* | *65.3* | *103.5* | *75.24* | | *113.39* | |
| rMOKV | 4-1 | < 0.2 [a] | < 4 | < 4 | < 4 | 0.6 [c] | < 4 | 2.12 [c] | SPBN | 1.0 [c] | |
| | 4-2 | | < 4 | < 4 | < 4 | > 0.2 [c] | < 4 | 0.4 [c] | | *Succumbed* | |
| | 4-3 | | < 4 | < 4 | < 4 | > 0.2 [c] | < 4 | 0.35 [c] | | *Succumbed* | *2.03* |
| | 4-4 | | < 4 | < 4 | < 4 | > 0.2 [c] | < 4 | 0.4 [c] | | 1.4 [c] | |
| | 4-5 | | < 4 | < 4 | < 4 | > 0.2 [c] | < 4 | 2.95 [c] | | 3.7 [c] | |
| | 4-6 | | < 4 | < 4 | < 4 | 2.0 [c] | < 4 | 2.46 [c] | rMOKV | 1.4 [c] | |
| | 4-7 | | < 4 | < 4 | < 4 | > 0.2 [c] | < 4 | 0.4 [c] | | 0.7 [c] | |
| | 4-8 | | < 4 | < 4 | < 4 | > 0.2 [c] | < 4 | 0.42 [c] | | 1.2 [c] | *0.88* |
| | 4-9 | | < 4 | < 4 | < 4 | > 0.2 [c] | 8.2 | 33.96 [c] | | 0.9 [c] | |
| | 4-10 | | < 4 | < 4 | < 4 | > 0.2 [c] | *No data* | > 0.2 [c] | | < 0.2 [c] | |
| | *Avg.* | | | | | | | *4.37* | | *1.31* | |

All values listed in IU/ml.

*a* Sera pooled, 1:5 starting dilution (LOD 0.2 IU/ml)

*b* Sera pooled, 1:50 starting dilution (LOD 4 IU/ml)

*c* Sera tested in singlet, 1:5 starting dilution (LOD 0.2 IU/ml)

**Table S1. RABV neutralizing titers.** Related to Figure 4. RABV neutralizing titers in IU/ml as determined by RFFIT. The 4 immunogen groups are labeled in the first column: mock immunization with PBS, and immunization with rRABV, rMOKV, and LyssaVax. On day 58 after the start of immunizations, 5 mice from each group were challenged with either live SPBN or live rMOKV. Level of detection (LOD) ranged from 0.2 IU/ml to 4 IU/ml based on starting serum dilution (1:5 to 1:50, respectively). All samples tested in duplicate except individual serum samples tested 1:5, which were tested in singlet.

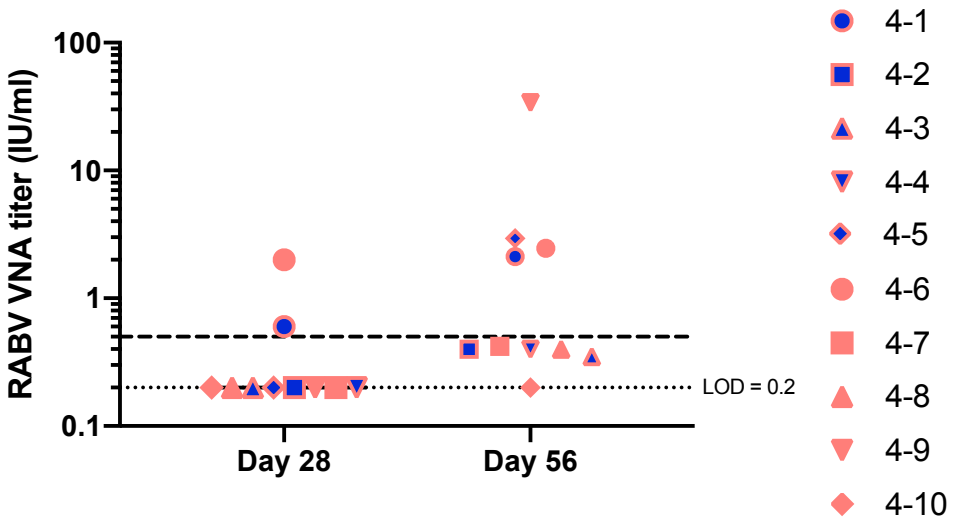


**Figure S6. Lower threshold of RABV neutralizing titers in sera from rMOKV-immune mice.** Related to Figure 4. RABV neutralizing antibody titers in individual mice (n=10 per group) at days 28 and 56 after immunization with rMOKV. See **Fig. 3A** for full immunization scheme. Red symbols indicate mice which were later challenged with rMOKV. Symbols with blue interiors indicate mice which were later challenged with SPBN. Titers were calculated in international units (IU) per ml by comparison to the U.S. standard rabies immune globulin. Level of detection (LOD) was 0.2 IU/ml (dotted line). Dashed line at 0.5 IU/ml indicates accepted level of RABV-neutralizing antibodies necessary for protection. Exact titers listed in Table S1.

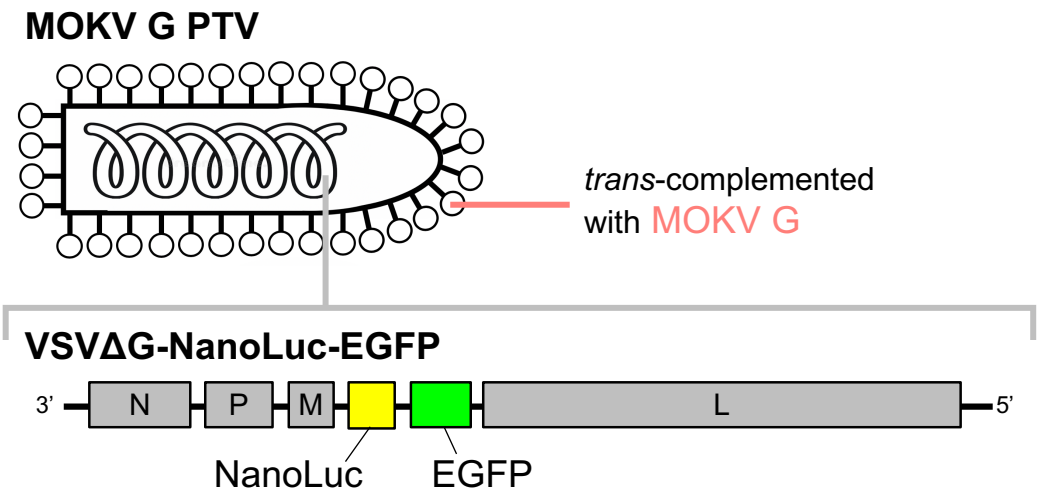


**Figure S7. Design of single-round VSV pseudotyped with MOKV G.** Related to Figure 5. MOKV G pseudotype viruses (PTVs) are single-round infectious virons which induce NanoLuciferase (NanoLuc) and EGFP expression in cells upon infection.

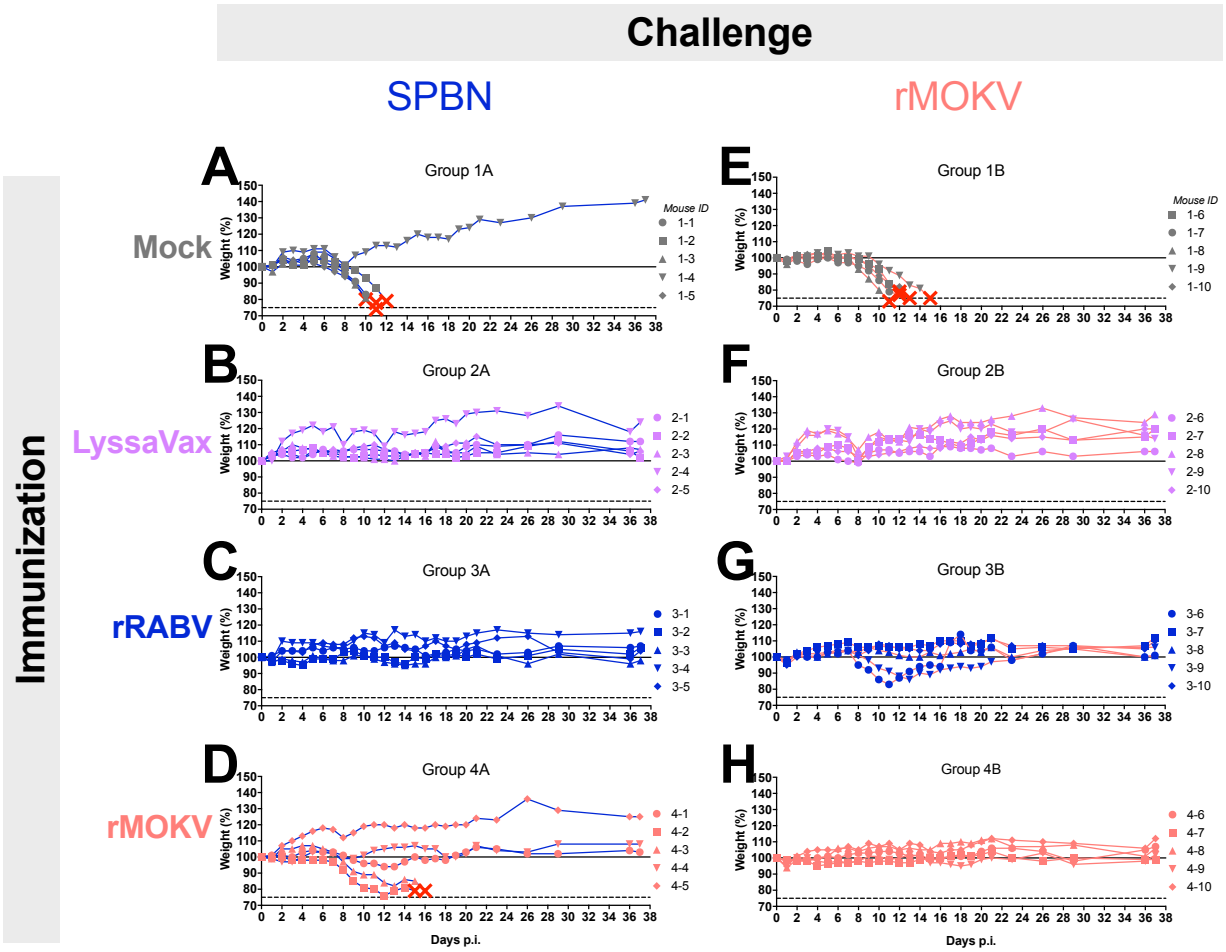


**Figure S8. Lower threshold of RABV neutralizing titers in sera from rMOKV-immune mice.** Related to Figure 6. Weight curves of mice immunized with either a mock vaccine (**A, E**), LyssaVax **(B, F)**, rRABV (**C, G**), or rMOKV (**D, H**)that were challenged i.n. with either $10^5$ FFU of live RABV (SPBN strain, **A-D**) or rMOKV (**E-H**) at day 58 post-immunization (p.i.). Mice which exhibited symptoms of disease or lost greater than 25% of day 0 weight were euthanized.