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Supporting Information for

Mucosal and systemic neutralizing antibodies to norovirus induced in infant mice orally inoculated with recombinant rotaviruses

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SI Materials and Methods

Cells and Viruses

MA104 cells (ATCC CRL-2378.1) were cultured in Medium 199 (gibco, cat. #11150-059) supplemented with 10% Fetal Bovine Serum (FBS), 0.292 mg/ml of L-glutamine, 100 I.U./ml of penicillin, and 100 µg/ml of streptomycin (CORNING, cat. #30-009-CI). BHK-T7 cells were kindly gifted from Dr. Buchholz (National Institute of Health) (1), and cultured in DMEM (CORNING, cat. #10-013-CV) supplemented with 10% FBS, 0.292 mg/ml of L-glutamine, 100 I.U./ml of penicillin, 100 µg/ml of streptomycin, and Geneticin (gibco, cat. #10131-035). Recombinant wild-type RRV was rescued as previously described and propagated in MA104 cells cultured in serum-free Medium 199 (SFM) in the presence of 0.5 µg/ml trypsin (Sigma-Aldrich, cat. #T0303) (2).

Recombinant proteins and antibodies

Recombinant HuNoV VLP (MD145 strain) and HuNoV P particles (VA387 strain) were generated as previously described (3, 4). The following antibodies were used in this study: anti-RV double-layered particle rabbit antiserum generated in Greenberg's lab, anti-RV triple-layered particle guinea pig antiserum generated in Greenberg's lab, anti-HuNoV VLP guinea pig antiserum (403 anti-MD2004 virus [GII.4]) (5), anti-HuNoV P particle rabbit antiserum (VA387 strain [GII.4] (6), anti-RV capsid protein VP6 mouse monoclonal antibody (Santa Cruz Biotechnology, clone 2B4, cat. #sc-101363), anti-β-actin mouse monoclonal antibody (Sigma-Aldrich, clone AC-74, cat. #A2228), HRP conjugated anti-mouse IgG goat polyclonal antibody (Sigma Aldrich, cat. #A4416), HRP conjugated anti-rabbit IgG goat polyclonal antibody (Sigma-Aldrich, cat. #A0545), HRP conjugated anti-guinea pig IgG goat polyclonal antibody (Sigma-Aldrich, cat. #A5545), Alexa Fluor488 conjugated anti-mouse IgG donkey polyclonal antibody (Jackson ImmunoResearch, cat. #715-545-150), Alexa Fluor488 conjugated anti-guinea pig IgG donkey polyclonal antibody (Jackson ImmunoResearch, cat. #706-545-148), Alexa Fluor594 conjugated anti-rabbit IgG donkey polyclonal antibody (Jackson ImmunoResearch, cat. #711-585-152). DyLight488 conjugated anti-mouse IgA goat polyclonal antibody (Abcam, cat. # ab97011). Rabbit antiserum to RV NSP3 was kindly gifted by Dr. Didier Poncet (Université Paris-Saclay) (7).

Plasmid construction

The plasmid encoding African swine fever virus (ASFV) capping enzyme and T7 RNA polymerase (C3P3-G1) was kindly gifted by Dr. Philippe H. Jaïs (8). To construct the plasmid harboring HuNoV VP1 or P domain in RV gene 7 (pT7-RRV-NSP3-HuNoV-VP1 and pT7-RRV-NSP3-HuNoV-P), the gene (ORF2) encoding the VP1 sequence of the HuNoV Sydney strain or P domain of the HuNoV VA387 strain were amplified by PrimeStar HS DNA polymerase (Takara Bio, cat. #r010a) and replaced the sequence encoding GFP in the pT7-RRV-NSP3-GFP constructed previously (2) by NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, cat. #E2621S). To construct expression vectors encoding HuNoV-VP1 (pCAG-HuNoV-VP1) and RV-VP6 (pCAG-RV-VP6), the ORF2 of HuNoV Sydney strain VP1 or RV-VP6 were cloned in the pCAG vector by NEBuilder HiFi DNA Assembly Master Mix, respectively. The plasmid sequences were confirmed by DNA sequencing.

Reverse genetics

Recombinant viruses were rescued by previously describe reverse genetics protocol with minor modification (2). Briefly, eight rescue plasmids (pT7-RRV-VP1, VP2, VP3, VP4, VP6, NSP1, NSP3, and NSP4) (0.4 µg of each), two rescue plasmids (pT7-RRV-NSP2 and NSP5) (1.2 µg of each) and C3P3-G1 (0.8 µg) were mixed with Trans-IT LT1 transfection Reagent (12.8 µl) (Mirus Bio, cat. #MIR2305) in OPTI-MEM I Reduced Serum Medium (150 µl) (Thermo Fisher Scientific, cat. #31985062). The mixture was transfected into BHK-T7 cells seeded in 12 well plates. The medium was replaced with serum-free DMEM 24 hours post-transfection. MA104 cells (1×10^5 cells) were overlayed on the BHK-T7 cells 48 hours post-transfection and cultured for three days in the presence of 0.5 µg/ml of trypsin. The cells were frozen/thawed and passaged in MA104 cells. To rescue rRRV-HuNoV-VP1 and rRRV-HuNoV-P, pT7-RRV-NSP3-HuNoV-VP1 or pT7-RRV-NSP3-HuNoV-P was used in place of pT7-RRV-NSP3. The rescued virus was propagated with MA104 cells in SFM in the presence of 0.5 µg/ml trypsin.

RNA-PAGE analysis of viral dsRNA and genetic stability
rRRV-HuNoV-VP1 and rRRV-HuNoV-P was passaged in MA104 cells by infection at an MOI of ~1 FFU/cell. Viral dsRNAs were extracted from passage 3 stock of rRRV, passages 3, 4, and 5 stocks of rRRV-HuNoV-VP1, and passages 3, 4, 5, and 6 stocks of rRRV-HuNoV-P with Trizol Reagent (Thermo Fisher Scientific, cat. #15596018) according to the manufacturer's instruction. The extracted dsRNA was mixed with Gel Loading Dye Purple (6×) (New England Biolabs, cat. #B7024S) and separated by 10% polyacrylamide gel (25 mA at 4°C for 18 hours), then stained by silver staining.

Growth kinetics
MA104 cells (seeded on 24 well plates) were infected with rRRV, rRRV-HuNoV-VP1, or rRRV-HuNoV-P at MOI of 1 or 0.01 FFU/cell. The viruses were activated with 5 μg/ml of trypsin at 37°C for 15 minutes. One hour after inoculation, the inoculum was removed, and the cells were washed with the SFM twice and cultured with the SFM in the presence of 0.5 μg/ml of trypsin. The cells were frozen at individual time points. Virus titer in the cells was determined by focus assay.

Virus titration by focus forming unit (FFU)
Sample viruses were activated by 5 μg/ml of trypsin at 37°C for 15 minutes and serially diluted in SFM. MA104 cells (seeded on 96 well plates) were infected with the diluted samples. One hour after inoculation, the inoculum was removed and the cells were washed with SFM once, then cultured with SFM for 14 hours. The cells were fixed with 10% formalin (Fisherbrand, cat. #23-245685), permeabilized with PBS containing 0.05% Tween-20, and stained with rabbit anti-RV antiserum (dilution 1:1,000) at room temperature for 2 hours. The cells were stained with HRP conjugated anti-rabbit IgG at 4°C overnight. RV-positive cells were visualized by AEC Substrate Kit, Peroxidase (Vector Laboratories, cat. #SK-4200) and counted under a microscope. The virus titer was expressed as FFU/ml.

Immunostaining
BHK-T7 cells (seeded on 96 well plates) were transfected with either pT7-RRV-NSP3 with C3P3-G1, pCAG-HuNoV-VP1, or pT7-RRV-NSP3-HuNoV-VP1 (100 ng/well) by TransIT-LT1 and cultured for 36 hours. MA104 cells (seeded on 48 well plates) were infected with rRRV or rRRV-HuNoV-VP1 at an MOI of 1 FFU/cell and cultured for 24 hours or were transfected with pCAG-HuNoV-VP1 (250 ng/well) by TransIT-LT1 and culture for 3 days. The BHK-T7 cells and MA104 cells were fixed with 10% formaldehyde, permeabilized with PBS containing 0.1% triton-X, and blocked with PBS containing 2% FBS. The cells were stained with rabbit anti-RV NSP3 (dilution 1:1,000) and guinea pig 403 anti-HuNoV MD2004 virus (GII.4) VLP (dilution 1:1,000) at 37°C for 2 hours followed by Alexa488 conjugated anti-guinea pig IgG (dilution 1:1,000) and Alexa594 conjugated anti-rabbit IgG (dilution 1:1,000) at 37°C for 2 hours. The nuclei were stained with 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI; Thermo Fisher Scientific, cat. #D3571). The images were acquired using an All-in-One Fluorescence Microscope BZ-X710 (Keyence).

Western blotting
MA104 cells (seeded on 24 well plates) were infected with rRRV, rRRV-HuNoV-VP1, or rRRV-HuNoV-P at an MOI of 4 FFU/cell and cultured for 12 hours. At 12 hours post-infection, the cells were lysed with Laemmli sample buffer (Bio-Rad, cat. #1610737) and boiled at 95°C for 5 minutes. The proteins were resolved with 4-15% Mini-PROTEAN TGX Gel (Bio-Rad, cat. #456-1086) and transferred to the PVDF membrane (Bio-Rad, cat. #162-0177) and blocked with PBS-T containing 3% Nonfat Dry Milk (Cell Signaling Technology, cat. #9999S) at 4°C overnight. The proteins were reacted with guinea pig 403 anti-HuNoV MD2004 virus (GII.4) VLP (dilution 1:1,000), rabbit anti-HuNoV-P VA387 strain (dilution 1:1,000), rabbit anti-RV NSP3 (dilution 1:1,000), mouse anti-RV capsid protein VP6 (dilution 1:1,000), or anti-β-actin mouse monoclonal antibody (dilution 1:1,000) at room temperature for 2 hours, then reacted with appropriate HRP conjugated secondary antibodies. Protein expression was visualized with Supersignal West Femto Maximum Sensitivity

Substrate (Thermo Scientific, cat. #34095) and acquired by ChemiDoc MP Imaging System (Bio-Rad).

Mouse experiment

Wild type 129sv and *Stat1*$^{-/-}$ mice were originally purchased from Taconic Biosciences Inc. and maintained at the Veterinary Medical Unit of the Palo Alto VA Health Care System. To assess diarrhea rate and fecal RV shedding, 5-day-old 129sv or *Stat1*$^{-/-}$ pups were orally inoculated with rRRV or rRRV-HuNoV-VP1 (3.9×10^5 FFU/pup) and monitored for diarrhea following gentle abdominal pressure for 10 days. We regarded as positive for diarrhea if liquid or unformed stools were observed as previously described (9). Stool samples were collected in 40 µl of PBS (+) (CORNING, Cat. #21-030-CV) and stored at -80°C until use. To evaluate serum and fecal antibody titers against RV and HuNoV VLPs, 5-day-old 129sv or *Stat1*$^{-/-}$ pups were orally inoculated with rRRV-HuNoV-VP1 (3.9×10^5 FFU/pup) and intraperitoneally injected with rRRV-HuNoV-VP1 (3.9×10^5 FFU/pup) at 9 weeks post-inoculation (WPI). Blood and stool samples were collected at 4, 6, 8, and 10 WPI. The immunogenicity of rRRV-HuNoV-VP1 was confirmed by two independent experiments. Representative data of serum and mucosal antibody responses are shown in Figures 3C, 4C, and 4D. To evaluate serum and fecal antibody titers against RV and HuNoV P particles, 5-day-old 129sv or *Stat1*$^{-/-}$ pups were orally inoculated with rRRV-HuNoV-P (2.0×10^5 FFU/pup) and intraperitoneally injected with rRRV-HuNoV-P (2.0×10^5 FFU/pup) at 5 weeks post-inoculation (WPI). Blood and stool samples were collected at 4 and 6 WPI. Collected blood was centrifuged at 2,300 ×g for 5 minutes and the supernatant was used as serum samples. Stool samples were collected in pre-weighed 1.5 mL tubes and PBS (+) was added to make 10% (w/v) of fecal suspension and stored at -80°C until use. The animal experiment protocol was approved by Stanford Institutional Animal Care Committee.

ELISA for detection of fecal RV antigens

RV fecal shedding was determined by sandwich ELISA. Briefly, ELISA plates (E&K Scientific Products, cat. #EK-25061) were coated with guinea pig anti-RV TLP antiserum (dilution 1:4,000), blocked with PBS containing 2% BSA (Sigma Aldrich, cat. #A7030) and incubated with RRV-infected MA104 cell lysate. After washing, 70 µl of PBS and 2 µl of fecal suspension were added to the plate and incubated at 4°C overnight. The amount of RV antigen was detected by rabbit anti-RV DLP antiserum (dilution 1:5,000) and peroxidase substrate (SeraCare, cat. #5120-0047). The OD450nm was detected by a microplate reader ELx800 (BIO-TEK).

Immunostaining for detection of serum IgG or fecal IgA against RV and HuNoV VP1

BHK-T7 cells (seeded on 96 well plates) were transfected with pCAG-HuNoV-VP1 or pCAG-RV-VP6 (100 ng/well). Two days after transfection, the cells were fixed with 10% formaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and blocked with PBS containing 2% FBS. The cells were stained with mouse sera (dilution 1:300) or fecal suspension (5% [w/v]) collected from virus-infected 129sv or *Stat1*$^{-/-}$ mice and stained with Alexa488 anti-mouse IgG (dilution 1:1,000) or Alexa488 anti-mouse IgA (dilution 1:1,000). The images were acquired using an All-in-One Fluorescence Microscope BZ-X710 (Keyence).

ELISA for detection of serum IgG and fecal IgA against RV, HuNoV VLP, and HuNoV P particle

To detect serum IgG and fecal IgA against RV, a sandwich ELISA was performed. ELISA plate was coated with rabbit anti-RV DLP antiserum (dilution 1:2,500), blocked with PBS containing 2% BSA, and incubated with RRV-infected MA104 cell lysate. After washing, the plates were incubated with serially diluted mouse sera or diluted fecal suspension (1% [w/v] for stools from 129sv, and 0.1% [w/v] for stools from *Stat1*$^{-/-}$) at 4°C overnight. To detect serum IgG and fecal IgA against HuNoV VLP and P particle, direct ELISA was performed. ELISA plate was coated with HuNoV VLP (150 ng/well) or HuNoV P (50 ng/well) and blocked with PBS containing 2% BSA. the plates were incubated with serially diluted mouse sera or 10% (w/v) of fecal suspension at 4°C overnight. The amount of serum IgG or fecal IgA was detected by HRP conjugated anti-mouse IgG (dilution 1:20,000) or HRP conjugated anti-mouse IgA (dilution 1:2,000) and visualized with peroxidase substrate. The OD450nm was detected by a microplate reader ELx800. For serum IgG time course,

the titer was determined as the highest dilution at which OD score is higher than that from uninfected mouse serum.

Blocking activities of mouse sera in the binding of HuNoV P particles to HBGA

Blocking activities of serum samples in the binding of HuNoV P particles to HBGA were determined by a saliva-based assay as previously described (6). The blocking antibody levels determined in this assay are considered to be a surrogate marker for neutralizing activity (10). Briefly, 96 well plates were coated with a well-characterized saliva sample and blocked with nonfat milk. This saliva was obtained from an Asian secretor donor who was positive for B, H2, Lewis b, and Lewis y HBGAs, but negative for A, H1, Lewis a, and Lewis x antigen (11). It bound strongly to GII.4 P particle (VA387) and was used for similar assays to measure blocking activities of human clinical sera in the binding of HuNoV P particles to HBGAs (12). Serially diluted mouse sera were mixed with P particle (GII.4 VA387 strain) (0.5 ng/µl) and added to the 96 well plates. The amount of bound P particle was detected by anti-HuNoV VLP rabbit antiserum (dilution 1: 3,300) followed by HRP conjugated anti-rabbit IgG. Fifty % blocking titer was determined as the highest dilution at which OD score is less than 50% of the OD score by P particle alone (no serum).

RV neutralizing assay

Serum and fecal samples were serially diluted in SFM and mixed with 400 FFU/100 µl of RRV and incubated at 37°C for 1 hour. MA104 cells (seeded on 96 well plates) were infected with 100 µl of the mixture of RRV with serum or fecal suspension and incubated at 37°C for 1 hour. After one hour of incubation, the cells were washed with SFM and incubated with SFM for 14 hours. The virus titer was determined as described in the virus titration section. RV neutralizing activities were determined as the percentage reduction in the number of foci compared to RRV alone (no serum). The anti-RV neutralizing titer in the sera was defined by greater than or equal to 50% focus reduction neutralizing test titer ($FRNT_{50}$).

Human intestinal organoids (HIOs) cultures

Adult, secretor positive, HIO cultures (J2 cell line) derived from jejunal biopsies were kindly provided by Dr. Mary Estes, Baylor College of Medicine (13, 14). Cultures were grown and maintained as described previously (15). Briefly, HIOs were suspended in 20 µl of Matrigel™ (Corning, cat# 356231) and grown as undifferentiated 3D cultures in 500 µL IntestiCult™ Organoid Growth Medium (OGM) Human (STEMCELL™ Technologies, cat# 06010) supplemented with 10 µM Y-27632 (Sigma-Aldrich, cat# Y0503). Undifferentiated monolayers were produced by plating single cell suspensions obtained from 7 days old highly dense 3D cultures. Cells were resuspended in 100 µl of IntestiCult™ OGM Human with 10 µM Y-27632 and seeded in collagen IV (Sigma-Aldrich, cat#. C5533) pre-coated 96 well plates. After 24 hours at 37°C and 5% CO_2, culture medium was replaced with differentiation medium [50/50; Base media / IntestiCult™ OGM Human Basal Medium (STEMCELL™ Technologies, cat# 100-0190)]. to induce cell differentiation. Cells were differentiated for 4 days. The base media compromises Advanced DMEM/F-12 (Gibco, cat# 12634010) supplemented with 1% GlutaMAX (Gibco, cat# 35050079), 1% Penicillin/Streptomycin (Gibco, cat# 15140122), and 1% 1M HEPES (Gibco, cat# 15630080).

Human norovirus neutralizing assay

Serum and fecal samples were serially diluted in infection media (base media supplemented with 500 µM sodium glycochenodeoxycholate (GCDCA; Sigma-Aldrich cat# G0759) plus 50 µM ceramide (C2; Santa Cruz Biotechnology, cat# sc-201375A) and mixed with 100 $TCID_{50}$/100 µl of GII.4 Sydney [P16] (GenBank # OL898515) and incubated at 37°C, for 1 hour. Differentiated HIE monolayers (seeded on 96 well plates) were infected with 100 µl of the mixture of HuNoV with serum or fecal suspension and incubated at 37°C, 5% CO_2 for 1 hour. After that, monolayers were washed with base media and incubated with differentiation media supplemented with 500 µM GCDCA plus 50 µM C2 for 24 hours. Plates were frozen are -70°C. Viral RNA was extracted from cultures (cells and media) using MagMAX™ - 96 Viral RNA Isolation Kit (Applied Biosystems, cat# AMB18365) according to the manufacturer's instructions. HuNoV RNA was detected by GI/GII TaqMan real-time RT-PCR (16). HuNoV neutralizing activities were determined as the percentage reduction in the number of genomic copies compared to HuNoV alone (no serum). The anti-HuNoV

neutralizing titer in the sera was defined by serum dilution that decreased HuNoV genome copy number by 50% (NT_{50}).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc.). Student's *t*-test was used for each time point in Figures 1B and 1C. One-way ANOVA was used for Figure 4F. Two-way ANOVA was used for Figures 2B, 2D 3B, 3C, and 5B. Neutralization titers (NT_{50}) were determined from log-transformed non-linear, dose-response sigmoidal curve fit data for Figures 3D and 3E.

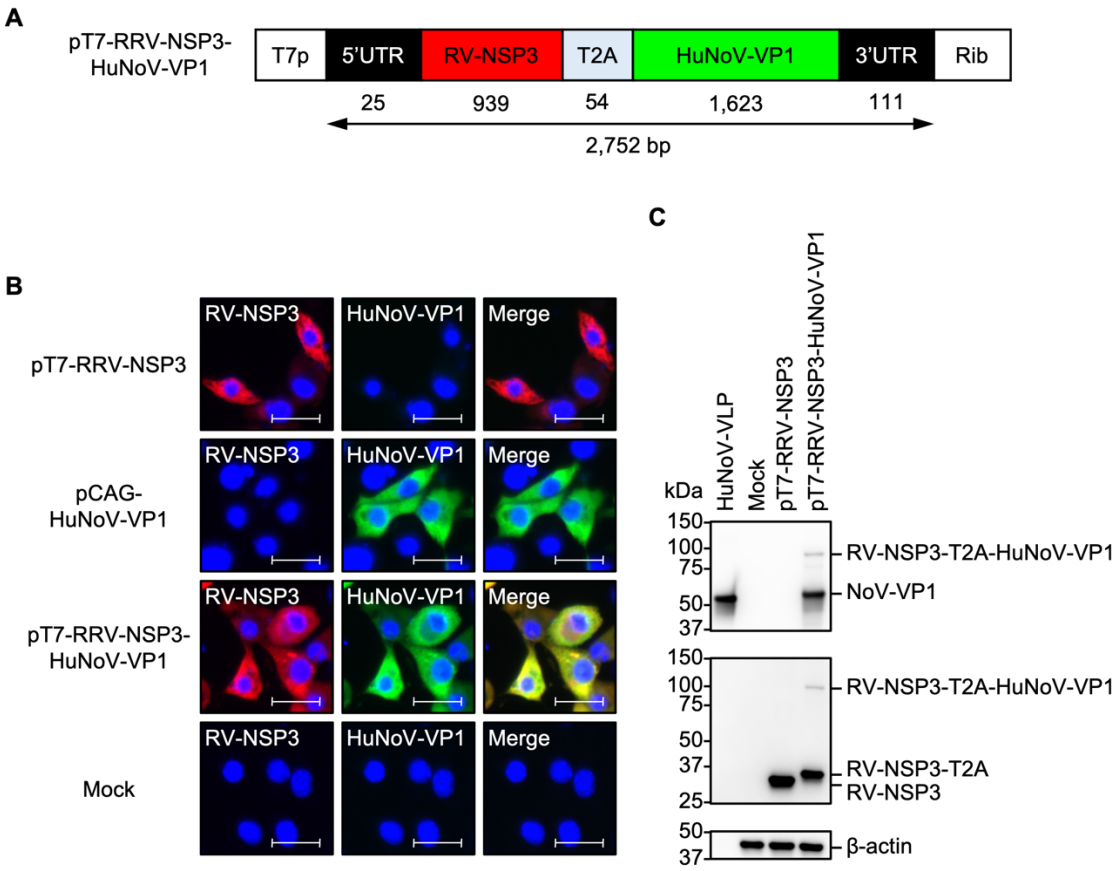

Figure S1. Strategy and validation of HuNoV VP1 expression from RV gene segment 7. (**A**) Schematic presentation of pT7-RRV-NSP3-HuNoV-VP1. T2A peptide and HuNoV capsid protein VP1 were inserted before the stop codon of RRV NSP3. The numbers indicate the nucleotide length of each gene cassette and untranslated regions (UTRs). The cDNA was flanked by the T7 promoter sequence (T7p) and the antigenomic hepatitis D virus ribozyme sequence (Rib). (**B**) Immunostaining analysis of protein expression by pT7-RRV-NSP3-HuNoV-VP1 in BHK-T7 cells. BHK-T7 cells were transfected with pT7-RRV-NSP3, pCAG-HuNoV-VP1, or pT7-RRV-NSP3-HuNoV-VP1 and fixed at 36 hours post transfection. The cells were stained with antibodies specific to RV-NSP3 (red), HuNoV-VP1 (green), and DAPI (blue). Scale bar: 25 μm. (**C**) Western blotting analysis of protein expression by pT7-RRV-NSP3 or pT7-RRV-NSP3-HuNoV-VP1 in BHK-T7 cells. BHK-T7 cells were transfected with pT7-RRV-NSP3 or pT7-RRV-NSP3-HuNoV-VP1 and harvested at 2 days post transfection. The cells were lysed with Laemmli buffer and resolved by SDS-PAGE. Protein expression of HuNoV-VP1, RV-NSP3, and β-actin was detected by the specific antibodies. The numbers show the molecular weights determined by the protein ladder.

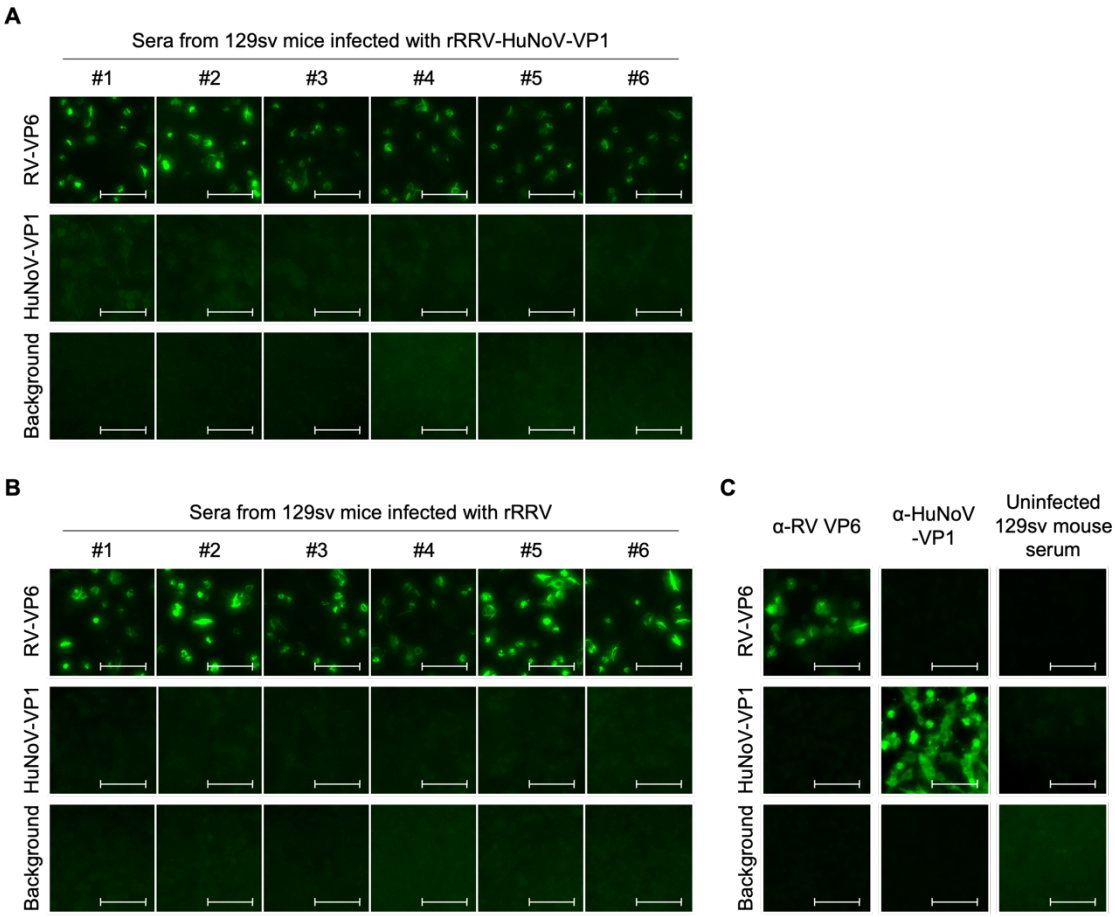

Figure S2. Detection of serum IgG against RV VP6 and HuNoV VP1 in sera from 129sv pups. (**A and B**) Immunostaining analysis with sera from 129sv pups inoculated with (**A**) rRRV-HuNoV-VP1 or (**B**) rRRV. BHK-T7 cells were transfected with pCAG-RV-VP6 or pCAG-HuNoV-VP1 and fixed at 2 days post transfection. The RV-VP6 and HuNoV-VP1 expressed in the cells were stained with indicated mouse serum and Alexa488 anti-mouse IgG. Scale bar: 100 μm. (**C**) Same as (**A and B**) except that the RV-VP6 and HuNoV-VP1 expressed in the cells were stained with control antisera and appropriate secondary antibodies. Scale bar: 100 μm.

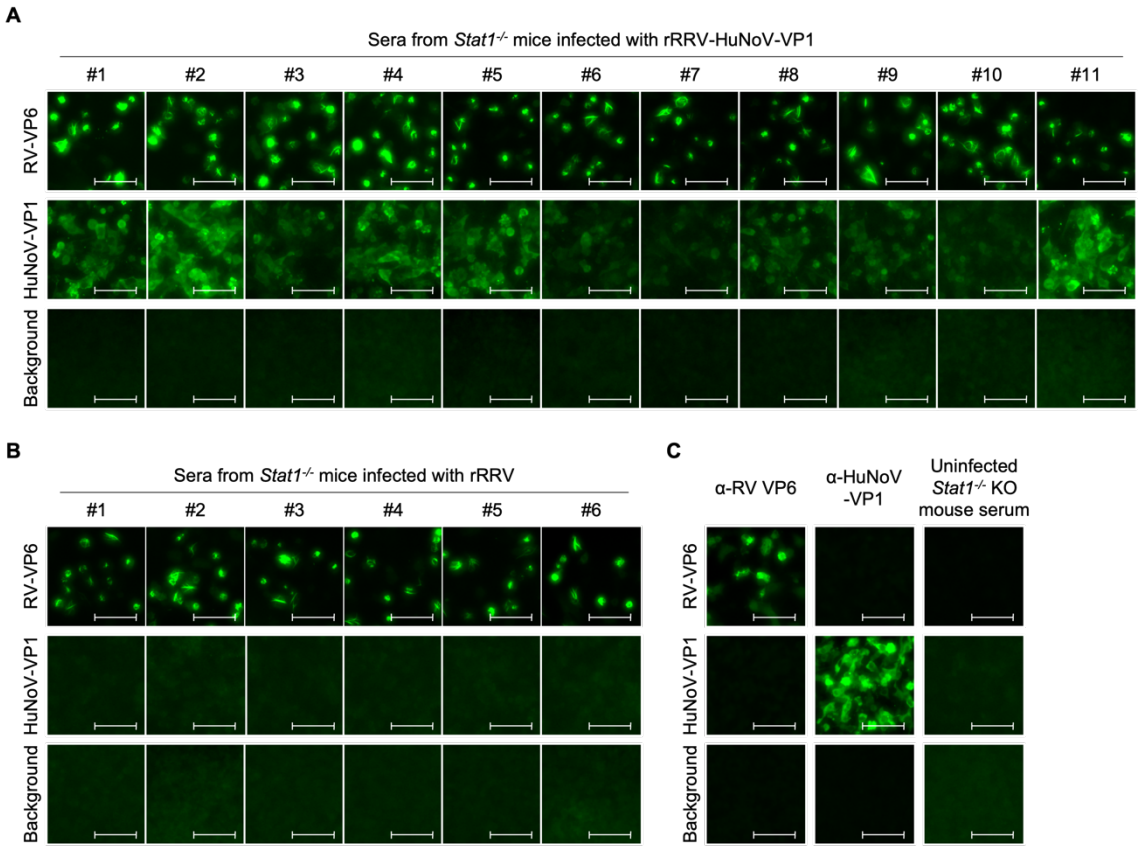

Figure S3. Detection of serum IgG against RV VP6 and HuNoV VP1 in sera from *Stat1*$^{-/-}$ pups. (**A and B**) Immunostaining analysis with sera from *Stat1*$^{-/-}$ pups inoculated with (**A**) rRRV-HuNoV-VP1 or (**B**) rRRV. BHK-T7 cells were transfected with pCAG-RV-VP6 or pCAG-HuNoV-VP1 and fixed 2 days post transfection. The RV-VP6 and HuNoV-VP1 expressed in the cells were stained with each mouse serum and Alexa488 anti-mouse IgG. Scale bar: 100 μm. (**C**) Same as (**A and B**) except that the RV-VP6 and HuNoV-VP1 expressed in the cells were stained with control antisera and appropriate secondary antibodies. Scale bar: 100 μm.

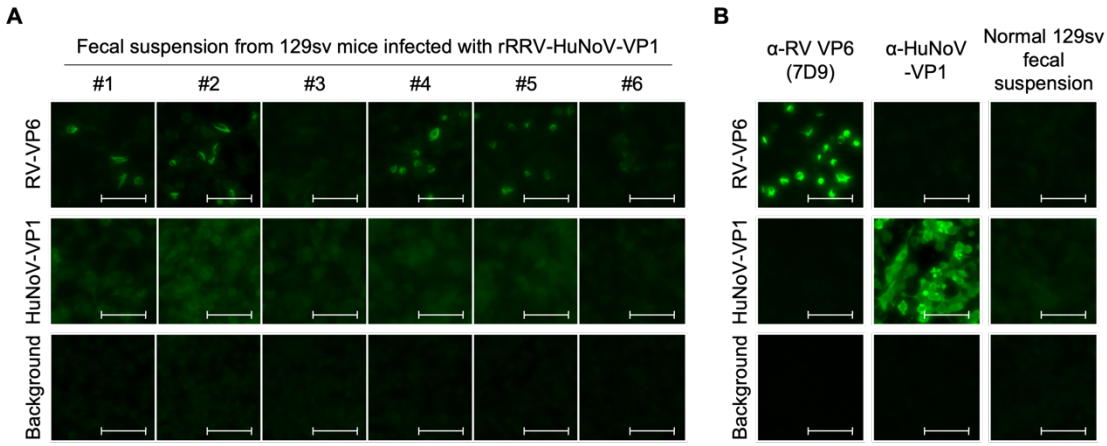

Figure S4. Detection of fecal IgA against RV VP6 and HuNoV VP1 in fecal specimens from 129sv pups.

(**A**) Immunostaining analysis with fecal supernatant from 129sv pups inoculated with rRRV-HuNoV-VP1. BHK-T7 cells were transfected with pCAG-RV-VP6 or pCAG-HuNoV-VP1 and fixed at 2 days post transfection. The RV-VP6 and HuNoV-VP1 expressed in the cells were stained with each mouse fecal supernatant and Alexa488 anti-mouse IgA. Scale bar: 100 μm. (**B**) Same as (**A**) except that the RV-VP6 and HuNoV-VP1 expressed in the cells were stained with control antisera and appropriate secondary antibodies. Scale bar: 100 μm.

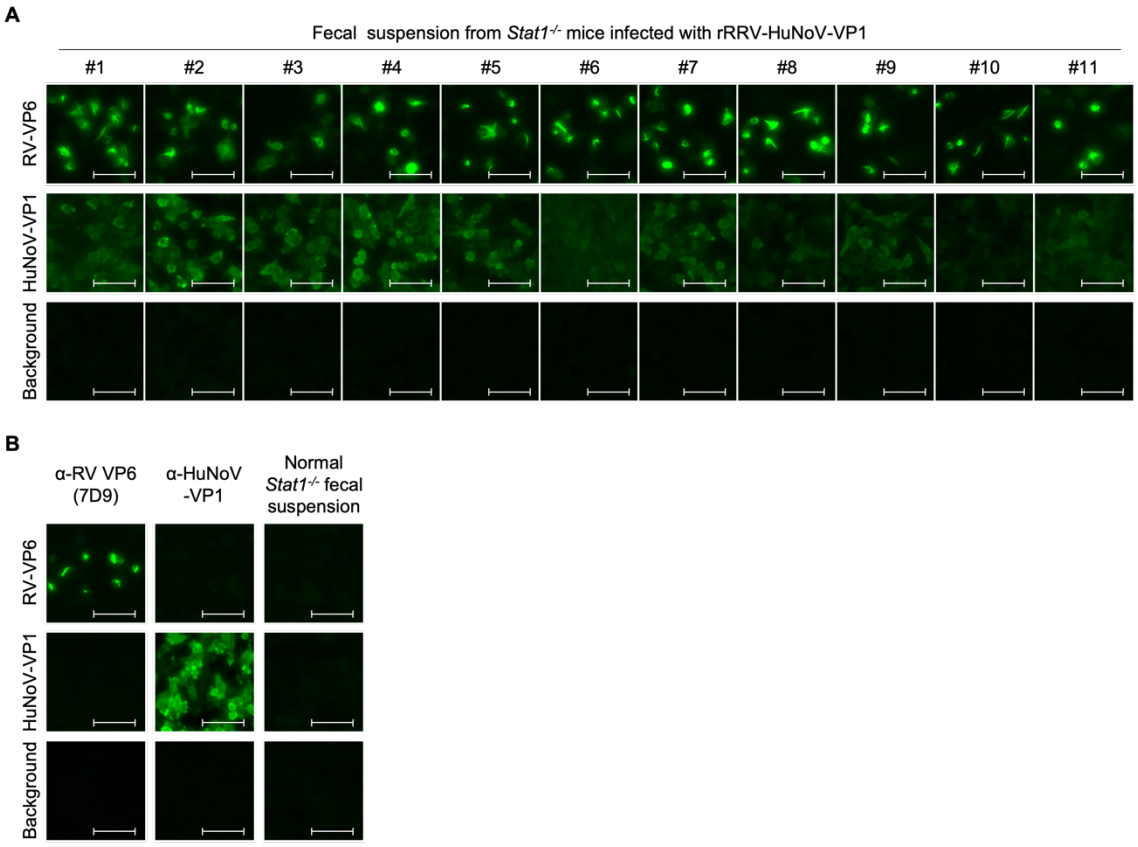

Figure S5. Detection of fecal IgA against RV VP6 and HuNoV VP1 in fecal specimens from *Stat1*$^{-/-}$ pups.

(**A**) Immunostaining analysis with fecal supernatant from *Stat1*$^{-/-}$ pups inoculated with rRRV-HuNoV-VP1. BHK-T7 cells were transfected with pCAG-RV-VP6 or pCAG-HuNoV-VP1 and fixed at 2 days post transfection. The RV-VP6 and HuNoV-VP1 expressed in the cells were stained with each mouse fecal supernatant and Alexa488 anti-mouse IgA. Scale bar: 100 μm. (**B**) Same as (**A**) except that the RV-VP6 and HuNoV-VP1 expressed in the cells were stained with control antisera and appropriate secondary antibodies. Scale bar: 100 μm.

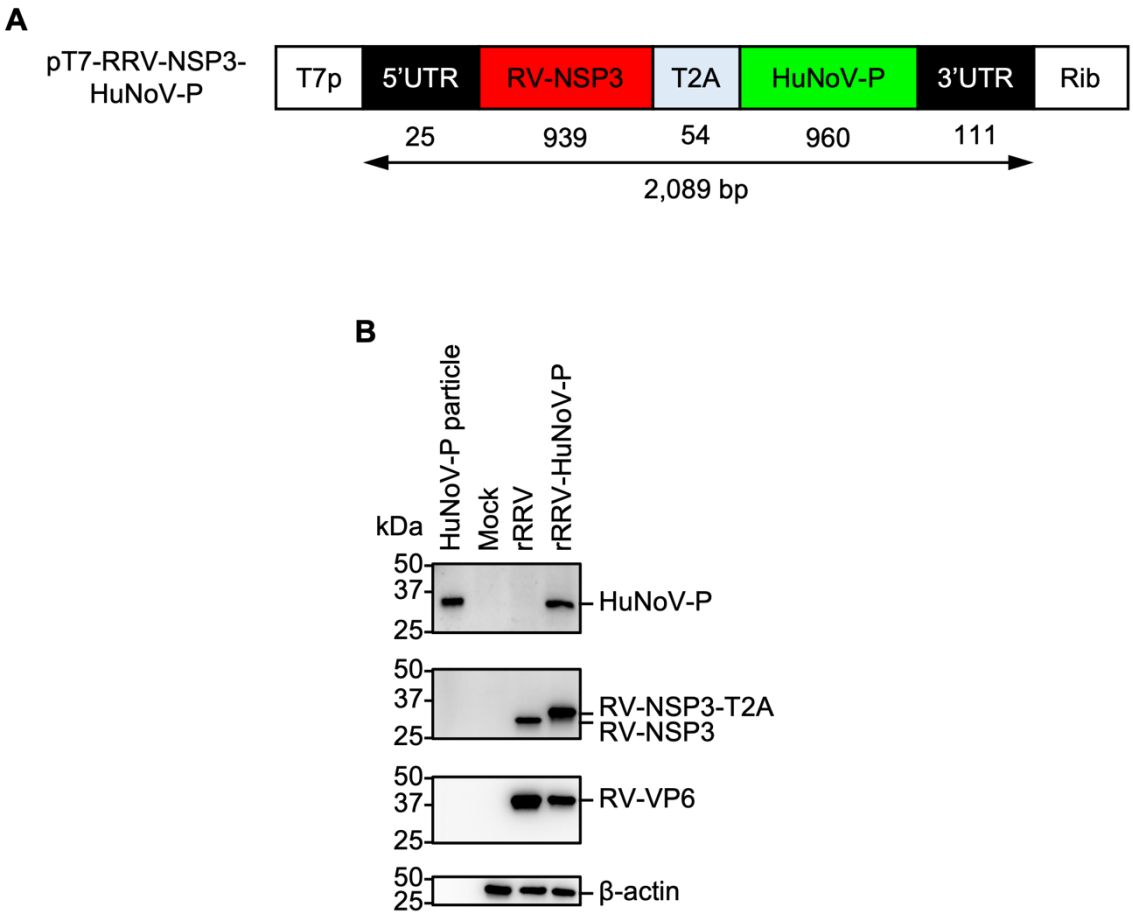

Figure S6. Generation of a recombinant RV expressing HuNoV P protein.
(**A**) Schematic presentation of pT7-RRV-NSP3-HuNoV-P. T2A peptide and HuNoV P domain were inserted before the stop codon of RRV NSP3. The numbers indicate the nucleotide length of each gene cassette and untranslated regions (UTRs). The cDNA was flanked by the T7 promoter sequence (T7p) and the antigenomic hepatitis D virus ribozyme sequence (Rib). (**B**) Western blotting analysis of protein expression by rRRV-HuNoV-P in MA104 cells. MA104 cells were infected with rRRV or rRRV-HuNoV-P at an MOI of 4 FFU/cell and harvested at 12 hours post infection. The cells were lysed with Laemmli buffer and resolved by SDS-PAGE. Protein expression of HuNoV-P, RV-NSP3, RV-VP6, and β-actin was detected by the specific antibodies. The numbers indicate the molecular weights (in kDa) of components of the protein ladder. Representative data of two independent experiments are shown.

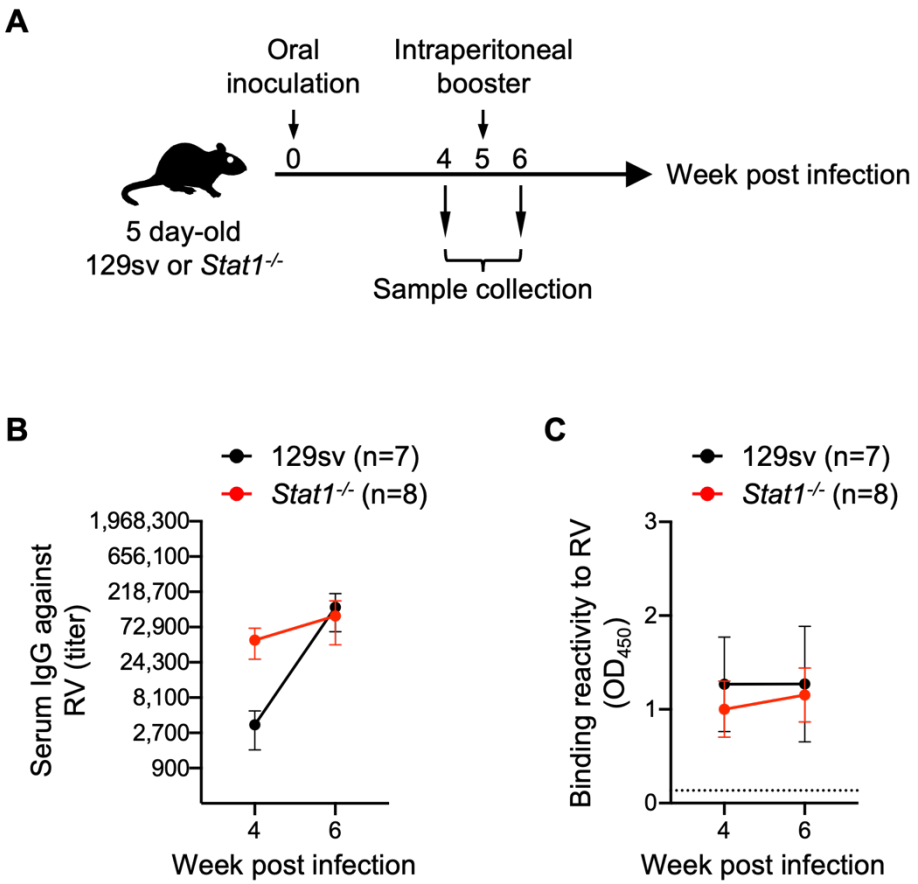

Figure S7. Systemic and mucosal antibody responses against RV in 129sv and Stat1-/- mouse following rRRV-HuNoV-P infection.
(**A**) Schematic presentation of the infection and immunization experiments. Five-day-old 129sv (n=7) or *Stat1*$^{-/-}$ (n=8) pups were orally inoculated with rRRV-HuNoV-P (2×10^5 FFU/pup) at 0 WPI and intraperitoneally boostered with the virus (2×10^5 FFU/pup) at 5 WPI. Blood and stool samples were collected at 4 and 6 WPI. (**B**) Temporal dynamics of serum IgG titers against RV. Mouse sera were serially diluted and tested in ELISA for anti-RV IgG. The IgG titer was defined as the highest serum dilution at which the OD score is higher than an uninfected mouse serum. Data are shown as mean with standard deviation. (**C**) Temporal dynamics of fecal IgA responses to RV. The amount of fecal IgA against RV in 1% (w/v) of fecal suspension from 129sv or 0.1% (w/v) of fecal suspension from *Stat1*$^{-/-}$ was determined by ELISA. Data are shown as the mean score of the net OD_{450} with standard deviation. The dotted lines show the limit of detection by stools from uninfected pups.

Table S1. Serum IgG responses against RV VP6 and HuNoV VLP in individual 129sv and *Stat1*$^{-/-}$ mice following rRRV-HuNoV-VP1 inoculation.

Animal ID	Serum IgG titer against RV		Serum IgG titer against HuNoV VLP	
	8 WPI	10 WPI (post booster shot)	8 WPI	10 WPI (post booster shot)
129sv #1	8,100	218,700	< 100	300
129sv #2	2,700	218,700	300	900
129sv #3	8,100	218,700	< 100	900
129sv #4	8,100	218,700	300	24,300
129sv #5	8,100	218,700	< 100	< 100
129sv #6	2,700	218,700	900	900
Average serum IgG titer (GMT)	5,616	218,700	114	736
Numbers of positive and total specimens	6/6	6/6	3/6	5/6
Stat1$^{-/-}$ #1	72,900	656,100	300	24,300
Stat1$^{-/-}$ #2	24,300	218,700	900	72,900
Stat1$^{-/-}$ #3	24,300	72,900	100	2,700
Stat1$^{-/-}$ #4	72,900	72,900	900	2,700
Stat1$^{-/-}$ #5	72,900	656,100	2,700	72,900
Stat1$^{-/-}$ #6	72,900	656,100	2,700	72,900
Stat1$^{-/-}$ #7	72,900	1,968,300	300	72,900
Stat1$^{-/-}$ #8	24,300	218,700	300	72,900
Stat1$^{-/-}$ #9	72,900	656,100	300	8,100
Stat1$^{-/-}$ #10	72,900	218,700	100	24,300
Stat1$^{-/-}$ #11	72,900	1,968,300	900	72,900
Average serum IgG titer (GMT)	54,026	398,196	494	26,852
Numbers of positive and total specimens	6/6	6/6	6/6	6/6

Table S2. Fecal IgA responses against RV VP6 and HuNoV VLP in individual 129sv and *Stat1*$^{-/-}$ mice following rRRV-HuNoV-VP1 inoculation.

Animal ID	ELISA OD_{450} score against RV [1]		ELISA OD_{450} score against HuNoV VLP [2]	
	8 WPI	10 WPI (post booster shot)	8 WPI	10 WPI (post booster shot)
129sv #1	0.836	2.171	0.574	0.507
129sv #2	1.034	1.672	1.241	1.737
129sv #3	0.387	2.064	0.143	0.184
129sv #4	1.196	1.338	0.17	0.351
129sv #5	1.002	0.754	0.08	0.3
129sv #6	0.822	0.849	0.744	1.505
Average ELISA OD_{450} score (Mean)	0.879	1.474	0.492	0.764
Numbers of positive and total specimens	6/6	6/6	5/6	6/6
Stat1$^{-/-}$ #1	1.688	1.463	0.208	0.154
Stat1$^{-/-}$ #2	1.71	1.802	0.155	3.042
Stat1$^{-/-}$ #3	0.889	0.863	0.105	0.106
Stat1$^{-/-}$ #4	1.64	1.696	3.261	3.206
Stat1$^{-/-}$ #5	0.775	0.863	0.831	2.645
Stat1$^{-/-}$ #6	1.013	0.776	0.173	1.738
Stat1$^{-/-}$ #7	1.588	1.265	0.524	2.876
Stat1$^{-/-}$ #8	0.921	0.478	0.197	0.658
Stat1$^{-/-}$ #9	0.694	0.506	0.095	0.258
Stat1$^{-/-}$ #10	0.345	0.348	0.165	0.699
Stat1$^{-/-}$ #11	1.727	1.922	0.175	1.6
Average ELISA OD_{450} score (Mean)	1.181	1.089	0.535	1.544
Numbers of positive and total specimens	11/11	11/11	9/11	10/11

[1] Supernatant of 1 % (w/v) of fecal suspension collected from 129sv and 0.1 % (w/v) of fecal suspension collected from *Stat1*$^{-/-}$ mice was used.
[2] Supernatant of 10 % (w/v) of fecal suspension collected from 129sv and *Stat1*$^{-/-}$ mice was used.

Table S3. Serum IgG responses against RV VP6 and HuNoV P particles in individual 129sv and *Stat1*$^{-/-}$ mice following rRRV-HuNoV-P inoculation.

Animal ID	Serum IgG titer against RV		Serum IgG titer against HuNoV P particles	
	4 WPI	6 WPI (post booster shot)	4 WPI	6 WPI (post booster shot)
129sv #7	2,700	218,700	<100	<100
129sv #8	2,700	218,700	<100	300
129sv #9	8,100	72,900	<100	<100
129sv #10	2,700	72,900	<100	<100
129sv #11	2,700	72,900	<100	<100
129sv #12	2,700	72,900	<100	<100
129sv #13	2,700	218,700	<100	<100
Average serum IgG titer (GMT)	3,158	116,736	<100	45
Numbers of positive and total specimens	7/7	7/7	0/7	1/7
Stat1$^{-/-}$ #12	24,300	72,900	900	8,100
Stat1$^{-/-}$ #13	72,900	72,900	100	2,700
Stat1$^{-/-}$ #14	72,900	72,900	300	8,100
Stat1$^{-/-}$ #15	72,900	218,700	300	24,300
Stat1$^{-/-}$ #16	24,300	218,700	<100	300
Stat1$^{-/-}$ #17	24,300	72,900	300	24,300
Stat1$^{-/-}$ #18	72,900	72,900	300	2,700
Stat1$^{-/-}$ #19	24,300	24,300	100	2,700
Average serum IgG titer (GMT)	42,088	83,631	198	4,676
Numbers of positive and total specimens	8/8	8/8	7/8	8/8

Table S4. Fecal IgA responses against RV VP6 and HuNoV P particles in individual 129sv and *Stat1*$^{-/-}$ mice following rRRV-HuNoV-P inoculation.

Animal ID	ELISA OD_{450} score against RV [1]		ELISA OD_{450} score against HuNoV P particles [2]	
	4 WPI	6 WPI (post booster shot)	4 WPI	6 WPI (post booster shot)
129sv #7	1.61	1.83	0.366	0.264
129sv #8	1.272	1.992	0.256	0.346
129sv #9	0.564	0.384	0.301	0.256
129sv #10	1.664	1.27	0.42	0.355
129sv #11	0.597	0.501	0.324	0.324
129sv #12	1.81	1.428	0.381	0.416
129sv #13	1.362	1.488	0.265	0.344
Average ELISA OD_{450} score (Mean)	1.26	1.27	0.330	0.329
Numbers of positive and total specimens	7/7	7/7	0/7	0/7
Stat1$^{-/-}$ #12	0.415	0.778	0.428	0.369
Stat1$^{-/-}$ #13	1.131	1.225	0.254	0.698
Stat1$^{-/-}$ #14	0.787	1.134	0.876	0.526
Stat1$^{-/-}$ #15	1.438	0.748	0.339	1.816
Stat1$^{-/-}$ #16	0.989	1.612	0.19	0.663
Stat1$^{-/-}$ #17	1.123	1.131	0.226	2.809
Stat1$^{-/-}$ #18	1.041	1.207	0.209	0.332
Stat1$^{-/-}$ #19	1.094	1.393	0.181	1.527
Average ELISA OD_{450} score (Mean)	1.00	1.15	0.337	1.092
Numbers of positive and total specimens	8/8	8/8	2/8	6/8

[1] Supernatant of 1 % (w/v) of fecal suspension collected from 129sv and 0.1 % (w/v) of fecal suspension collected from *Stat1*$^{-/-}$ mice was used.
[2] Supernatant of 10 % (w/v) of fecal suspension collected from 129sv and *Stat1*$^{-/-}$ mice was used.

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