Online data supplement

# Human rhinovirus 1B exposure induces PI 3-kinase-dependent airway inflammation in mice

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METHODS

*Animals.* Female C57BL/6 mice (ages 6-8 weeks) were purchased from Charles River Laboratories (Wilmington, MA) and housed in a specific pathogen-free area within the animal care facility at the University of Michigan.

*Cell Culture.* 16HBE14o- human bronchial epithelial cells originating from bronchial epithelial tissue transfected with pSVori-, containing the origin-defective SV40 genome (E1), were provided by Dr. Steven R. White (University of Chicago). Cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 2mM of L-glutamine. HeLa cells and LA-4 mouse bronchial epithelial cells were obtained from American Type Culture Collection (Manassas, VA). LA-4 cells were grown in Ham's F12K medium with 2 mM L-glutamine and 5% FBS and supplemented with 1% penicillin-streptomycin.

*Generation of RV stocks.* RV1B was generated from an infectious cDNA clone, as described (E2). RV39 was obtained from American Type Culture Collection. Viral stocks were generated as previously described (E3). Briefly, HeLa cells were infected with RV until 80% of the cells were cytopathic. HeLa cell lysates were harvested and cellular debris pelleted by centrifugation (10, 000 x *g* for 30 min at 4°C). RV in HeLa cell lysates was concentrated and partially purified by centrifugation with a 100,000 MW cutoff Centricon filter (2,000 rpm at 4°C for 8 h; Millipore, Billerica, MA) (E4). Virus was titered by infecting confluent HeLa monolayers with serially diluted RV (range: undiluted to $10^{-9}$) and assessing cytopathic effect five days after infection. Fifty percent tissue culture infectivity doses ($TCID_{50}$) values were determined by the Spearman-Karber method (E5). In some experiments, purified RV1B was

UV-irradiated on ice for 60 min using an UVB CL-1000 cross-linker at 1200 μJ/cm$^2$ (E4). The efficiency of UV irradiation was confirmed by reverse transcriptase-PCR (see below).

*RV1B exposure.* Near confluent 16HBE14o- and LA-4 cells were serum starved overnight and infected with RV1B at an MOI of 1.0 for 1 hour at 33°C. After removal of viral inoculum, fresh media was added and cells were incubated at 33°C for the remainder of the experiment. Mice were anesthetized by intraperitoneal injection with ketamine (40 mg/kg) and xylazine (5 mg/kg) and intranasally inoculated with 50 μl of 1x10$^8$ $TCID_{50}$/ml RV1B or equal volume sham HeLa cell lysate. In experiments using the PI 3-kinase inhibitor LY294002 (Sigma Chemical, St. Louis), mice were anesthetized as described above and given 50 μl of 3 mg/kg LY294002 (or dimethyl sulfoxide vehicle) intranasally. One hour later, while still anesthetized, mice were intranasally inoculated with 20 μl of 2.5x10$^8$ $TCID_{50}$/ml RV1B or equal volume sham. Mice were euthanized 1-14 days post-inoculation.

*Immunoblotting*. 16HBE14o- cells were lysed, cellular proteins were resolved by 10% SDS-PAGE, and proteins transferred to a nitrocellulose membrane. Membranes were probed with antibodies against Ser$^{473}$ phospho-Akt and total Akt (Cell Signaling, Beverly, MA).

*Presence of viral RNA.* RNA was extracted from lungs of mice using Trizol reagent (Sigma Chemical) and analyzed for the presence of viral RNA by reverse transcriptase-PCR. cDNA was generated from 3 μg of total RNA as previously described (E6). Cellular RNA was also collected by nasal lavage. Mice were positioned upside-down, and the trachea was intubated with a 1.7-mm OD polyethylene catheter which was directed cephalad. 300 μM of PBS containing 5 mM EDTA was instilled over a 5 min period. PBS dripping from the nares of mice was collected in a tube containing 1 ml Trizol. Quantitative one-step real time PCR for positive-strand viral RNA was conducted using RV-specific primers and probes for RV (forward

primer: 5'-GTG AAG AGC CSC RTG TGC T-3'; reverse primer: 5'-GCT SCA GGG TTA AGG TTA GCC-3'; probe: 5'-FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA-3')(E7). Copy numbers of positive strand viral RNA were normalized to 18S RNA, which was similarly amplified using gene-specific primers and probes (E7). Finally, negative-strand (replicative) RV RNA was quantitated by two step RT-PCR as described for hepatitis C virus (E8). Briefly, 5 μg of total RNA was reverse transcribed with 2 μl of Superscript III (Invitrogen, 50 min at 55°C) using 2 pmol of primer complementary to the antisense strand of RV RNA and tagged with non-RV sequence at the 5' end (5'ggc cgt cgt cat ggt ggc gga taa CCC AGC AGT AGA ACC TT3'). RNA was then treated with a combination of RNaseA and RNaseH to destroy the remaining RNA. Real time PCR was carried out with 5 μl of cDNA, 300 nM of each forward primer (5' TGG CTT CAC ACC TCA TA 3') and tag (5' ggc cgt cgt cat ggt ggc gga taa 3') and 175 nM of gene specific probe (5'-FAM AAG AGT GTG CAG GCA GGCC AC TAMARA-3') using a universal Taqman PCR kit.. To determine copy number, we constructed a plasmid containing a PCR product from replicating virus using the forward primer 5'-TGGCTT CACACC TCAT-3' and the reverse primer 5'-TGATAT CGTTAC CCGCAA AGT-3'. This plasmid was used to create the standard curve for real time-PCR. Copy numbers of negative-strand viral RNA were normalized to 18S RNA, which was similarly amplified. Reactions were performed on an Eppendorf realplex$^2$ (Westbury, NY).

*Histology and immunofluorescence staining.* Lungs were inflated with 4% paraformaldehyde and fixed overnight with 10% buffered formalin. Paraffin-mounted 5 μM sections were stained with hematoxylin and eosin and examined by light microscopy. For immunofluorescence staining, lung sections were deparaffinzed in three washes of xylene and rehydrated in an ethanol gradient. To ummask the antigenic sites, slides were heated under

pressure for 1 min in 0.01 M citrate buffer (pH 6.0). The tissues were blocked for 2 h with 5% goat serum in Tris-buffered saline (TBS), pH 7.6. Sections were incubated with a mixture of 1:250 diluted RV1B antiserum (American Type Culture Collection) and immunohistochemistry-specific phospho-Akt diluted 1:50 (Cell Signaling, Beverly, MA) overnight at 4°C. Slides were washed and incubated for 2 h at room temperature with the appropriate Alexa-Fluor conjugated secondary antibody (Molecular Probes, Portland, OR) diluted 1:1000. Coverslips were mounted with ProLong Antifade reagent (Molecular Probes), and visualized by confocal fluorescent microscopy using a Zeiss LSM 510 confocal microscope mounted on a Zeiss Axiovert 100M inverted microscope.

*Determination of RV1B infectivity.* RV1B infectivity was assessed by homogenizing lungs from RV1B, UV-irradiated RV1B, or sham-inoculated mice 1-4 days post-exposure. Tissue was centrifuged to remove debris; supernatants were resuspended in MEM supplemented with 3% serum and 1% penicillin-streptomycin and overlayed onto confluent monolayers of HeLa cells. Five days post infection, HeLa cells were assessed for cytopathic effects. In addition, cellular RNA was extracted using Trizol reagent (Sigma Chemical) and analyzed for the presence of positive-strand viral RNA by quantitative one-step real time PCR, as described above.

*Lung inflammatory cells.* Bronchoalveolar lavage (BAL) was performed by exposing and intubating the trachea using a 1.7-mm OD polyethylene catheter, and instilling PBS containing 5 mM EDTA in 1-ml aliquots. Cytospins prepared from BAL cells and stained with Diff-Quick (Dade Behring, Newark, DE) and differential counts were determined. MPO activity, a surrogate for neutrophil number, was measured by colorimetic assay, as previously described (E9).

*Cytokine/chemokine expression.* For *in vitro* experiments, cells were grown to 80% confluence, serum starved for 24 hours, and then infected with RV1B for 1 hour. Inoculum was then replaced with serum free medium. Conditioned medium was collected 48 h post infection, centrifuged to remove cell debris, and then frozen at -80°C. Protein levels were measured by ELISA (R&D Systems, Minneapolis, MN). For *in vivo* experiments, lungs were frozen in liquid nitrogen. RNA was extracted using Trizol reagent (Sigma Chemical) and analyzed for the presence of interferon (IFN)-α, β and γ by quantitative two-step real time PCR using specific primers and probes. All primers were from IDT (Coralville, IA) and employed FAM as the fluorescent tag and TAMRA as a quencher. Sequences included, for IFN-α, forward primer 5'- CCA TCC CTG TCC TGA GTG -3', reverse primer 5'- CCA TGC AGC AGA TGA GTC CTT -3', probe 5'- /56-FAM/AGC TGA CCC AGC AGA TCC TGA ACA TC/36-TAMSp/ -3'; for IFN-β, forward primer 5'- GAC GGA GAA GAT GCA GAA GAG TTA C -3', reverse primer, 5'- CCA CCC AGT GCT GGA GAA -3', probe 5'- /56-FAM/TGC CTT TGC CAT CCA AGA GAT GC/36-TAMSp/ -3'; and for IFN-γ, forward primer 5'- TGG CTG TTT CTG GCT GTT AC -3', reverse primer 5'- TCC ACA TCT ATG CCA CTT GAG TT -3' and probe 5'- /56-FAM/TGC CAC GGC ACA GTC ATT GAA A/36-TAMSp/ -3'. The signal was normalized to GAPDH and expressed as fold-increase over sham. In addition, mouse lungs were homogenized in 1 ml PBS containing complete protease inhibitor cocktail buffer (Roche Applied Science, Indianapolis, IN) and centrifuged for 10,000 x *g* for 15 min. Cleared supernatant was stored at -70°C and protein levels were measured by ELISA.

*Measurement of respiratory system resistance.* Mice were anesthetized with sodium pentobarbital (6.5 mg/kg mouse) and intubated via cannulation of the trachea with a 20-gauge stub adapter cannula (Becton Dickson, Sparks, MD). Mechanical ventilation was performed

using a FlexiVent ventilator (Scireq, Montreal, Quebec, Canada) at 150 breaths/min with a tidal volume of 10 ml/kg body weight. Airway responsiveness was assessed by measuring maximum respiratory system resistance in response to increasing doses of nebulized methacholine.

*Data analysis.* SigmaStat computing software (SPSS, Chicago, IL) was used for data analysis. Data are represented as mean±SEM. Statistical significance was assessed by one- or two-way analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by the Student Newman-Keuls' multiple range test.

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