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Impact of Host Proteases on Reovirus Infection in the Respiratory Tract

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Virion uncoating is an essential early event in reovirus infection. In natural enteric infections, rapid proteolytic uncoating of virions is mediated by pancreatic serine proteases. The proteases that promote reovirus disassembly and cell entry in the respiratory tract remain unknown. In this report, we show that endogenous respiratory and inflammatory proteases can promote reovirus infection *in vitro* and that preexisting inflammation augments *in vivo* infection in the murine respiratory tract.

Mammalian orthoreovirus (reovirus) entry into cells is characterized by the stepwise disassembly of virions into two functional subvirion particles, a membrane penetration-competent infectious subvirion particle (ISVP) and a transcriptionally active core (19). In cell culture, entry is initiated by attachment of viral protein σ1 (44) to a cell surface carbohydrate (6, 20, 32) and junctional adhesion molecule A (7). Virions are then internalized by β1 integrin-mediated endocytosis (15, 29, 47, 48). Within the endocytic compartment, host proteases remove the outer capsid protein σ3, exposing μ1, the membrane penetration protein (4, 25, 58). In murine fibroblasts, virion disassembly is mediated by the acid-dependent cysteine proteases, cathepsins (Cats) L and B (4, 5, 28). In immune cells, acid-independent proteases, including Cat S and neutrophil elastase, can promote virion uncoating (34, 36).

After oral inoculation of newborn mice, reovirus virions are rapidly uncoated in the enteric tract by the secreted pancreatic serine proteases chymotrypsin (CHT) and trypsin (8, 13). Extracellular uncoating is required for reovirus adherence to Peyer's patch M cells and subsequent entry into intestinal tissue (3). In humans and animals, reoviruses are also associated with pulmonary infections (10, 22, 46, 51), but little is known about the proteases that mediate virion uncoating when infection is initiated by this route. In this study, we investigated the role of inflammatory and endogenous respiratory proteases in reovirus infection.

Type II transmembrane serine proteases (TTSPs) expressed in the human airway have been shown to activate membrane fusion and promote cell entry of a number of respiratory pathogens, including influenza virus, human metapneumovirus, and severe acute respiratory syndrome (SARS) coronavirus (11, 12, 16, 17, 18, 33, 55–57). To assess the capacity of a respiratory TTSP to promote reovirus uncoating, we incubated purified reovirus type 1 Lang virions with human airway trypsin-like protease (HAT) and analyzed the products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A). After 6 h of treatment, only 2% of the σ3 outer capsid protein remained associated with virions, as determined by NIH Image J quantitation. The particles resembled ISVPs generated by pancreatic serine proteases (14), although, unlike treatment with CHT, HAT treatment did not lead to significant cleavage of the underlying capsid protein μ1C. To assess the infectivity of the HAT-treated subviral particles (HAT-SVPs) and determine whether they require additional intracellular proteolysis, we measured viral yields in cultures of L929 mouse fibroblasts that were left untreated or treated with the broad-spectrum cysteine protease inhibitor E-64 for 3 h prior to infection. E-64 inhibits Cats L and B, which mediate virion disassembly in L929 cells. As expected, E-64 treatment inhibited virion infection in L929 cells, whereas CHT-generated ISVPs (CHT-ISVPs), which lack the σ3 capsid protein, replicated to high yields (Fig. 1B). Like CHT-ISVPs, HAT-generated subviral particles replicated to high yields in the presence and absence of the protease inhibitor. These results indicate that the HAT respiratory protease can productively uncoat reovirus virions.

We used an additional approach to test whether HAT and a second TTSP, TMPRSS2, could promote reovirus infection. Vero cells, which are permissive for reovirus infection but do not support efficient virion disassembly (35), were transfected with FLAG-tagged protease expression plasmids (16) or a similarly tagged control plasmid (9). At 30 h after transfection, the cultures were infected with reovirus virions. At 18 h postinfection, cells were fixed and analyzed for evidence of viral replication and expression of the FLAG antigen (Fig. 1C). In the transfected cultures, expression of either HAT or TMPRSS2 led to a significant increase in the number of reovirus-infected cells, whereas expression of FLAG-tagged herpes simplex virus protein ICP22 from the control plasmid did not. Levels of infection in protease gene-transfected cultures were comparable to those in mock-transfected cultures that had been infected with CHT-ISVPs (Fig. 1C). When we compared infection by CHT-ISVPs in cultures expressing TTSPs and in control-transfected cultures, we found no significant difference in σNS expression in these samples (data not shown). Together, our results reveal that endogenous respiratory TTSPs are capable of productively uncoating reovirus virions *in vitro* and of promoting infection in cell culture.

In vivo, HAT and TMPRSS2 are expressed on the membrane of bronchiolar epithelial cells (12, 21, 27, 49, 59) and have murine homologues, namely, MAT and epitheliasin (37, 38). However, the activity of these and other respiratory proteases is balanced by

Received 27 September 2011 **Accepted** 26 October 2011

Published ahead of print 9 November 2011

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doi:10.1128/JVI.06429-11

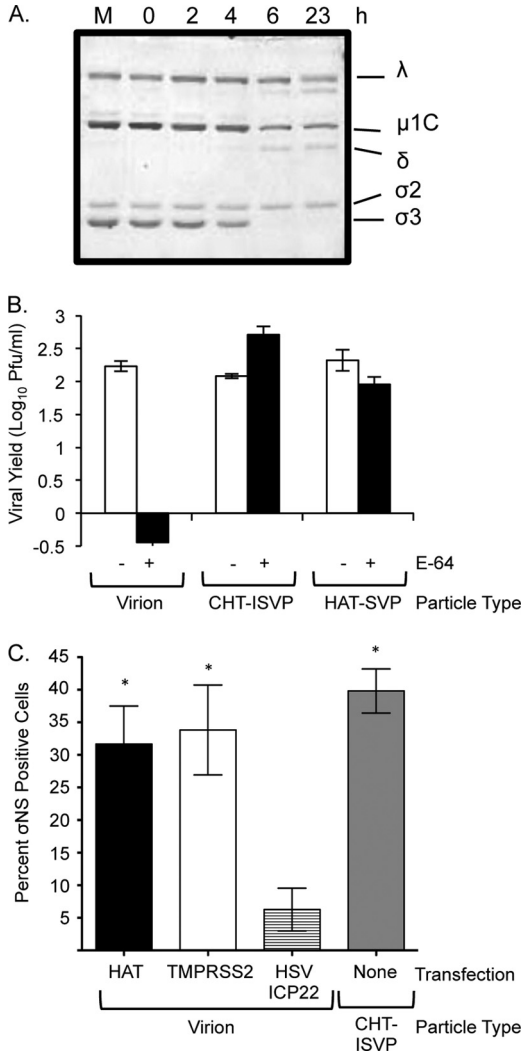

FIG 1 Effect of type II transmembrane serine proteases on reovirus virions and infectivity. (A) Reovirus strain Lang virions (5×10^{10}) in virion dialysis buffer (150 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris [pH 7.5]) were incubated with purified HAT (R&D Systems) (21 μg/ml) for the indicated times. Reactions were stopped with 5 mM benzamidine. The mock sample (M) consisted of virions held in reaction buffer in the absence of protease for 23 h. Treated particles were analyzed using SDS–12% polyacrylamide gels, and proteins were visualized by Coomassie staining. The positions of reovirus capsid proteins are labeled. (B) L929 cells were left untreated (white bars) or pretreated for 3 h with 300 μM E-64 (black bars) and infected with Lang virions, CHT-ISVPs, or HAT-SVPs at a multiplicity of infection (MOI) of 5 PFU/cell. Infections were carried out in the presence or absence of E-64 and terminated at 1 day postinfection. Viral yields were determined by plaque assay on L929 cells. Values represent the means ($\pm$ standard errors [SE]) of the results of experiments performed with triplicate samples. (C) Vero cell cultures were transfected with HAT, TMPRSS2, or HSV ICP22 expression plasmids 30 h prior to infection with reovirus strain Lang virions or CHT-ISVPs (MOI, 10 PFU/cell). At 18 h postinfection, cells were fixed and permeabilized with 4% paraformaldehyde. Indirect immunofluorescence was used to detect expression of the reovirus nonstructural protein σNS and the presence of the FLAG-tagged protease or control protein. Cells were counterstained with Hoechst stain to facilitate quantification. Reovirus-infected and transfected cells were quantified by counting fluorescent cells in a minimum of three fields in each of 3 independent experiments. Between 85 and 150 cells were counted per field. Data are presented as the percentage of cells expressing σNS relative to the number of FLAG-positive cells in the field. Error bars indicate SEM. *, $P < 0.05$ (Mann-Whitney test).

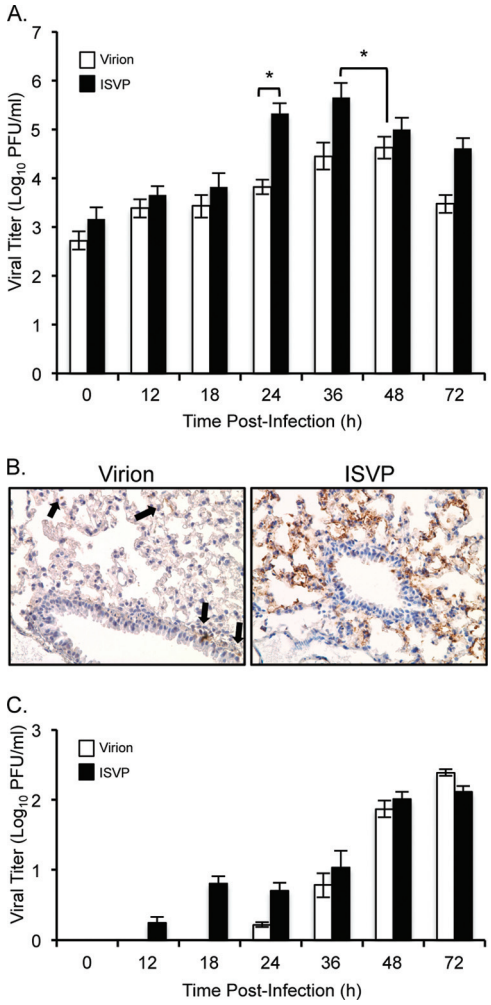

FIG 2 Comparative analysis of reovirus infection in the respiratory tract after intranasal inoculation with virions or ISVPs. CBA/J mice (4 weeks old) were inoculated intranasally with 1×10^7 PFU Lang virions or CHT-ISVPs. Organs were harvested at the indicated times postinoculation, and viral titers in the lungs (A) and spleen (C) were determined by plaque assay on L929 cells. Results are expressed as the means of viral titers for 6 to 12 mice per time point. Error bars indicate standard errors of the means (SEM). *, $P < 0.05$ (as determined by the Mann Whitney test). (B) At 24 hpi, lungs were isolated, sectioned, and stained with antiserum directed against the reovirus μNS nonstructural protein. Viral antigen was detected using a horseradish peroxidase-linked secondary antibody and diaminobenzidine, and slides were counterstained with hematoxylin. Representative sections are shown, and arrows point to some antigen-positive cells. Specificity of the staining was determined from control slides, which included infected lung sections incubated with preimmune serum and uninfected lung sections incubated with μNS-specific antiserum (data not shown).

the presence of locally produced protease inhibitors (31). To assess the efficiency of reovirus uncoating in the respiratory tract, we used a murine respiratory model (46) to compare viral loads after infection with virions or ISVPs. Four-week-old CBA/J mice were inoculated intranasally (10, 51) with type 1 Lang virions or ISVPs. Organs were harvested at various times postinoculation, and viral titers were determined by plaque assay on L929 cells. We observed equivalent lung titers at early times (12 h) following infection with virions or ISVPs (Fig. 2A), likely reflecting the inoculum. However, by 24 h postinoculation (hpi), we recovered significantly

higher titers in the lungs of ISVP-infected animals. Titers from the lungs of virion-infected animals increased more slowly. Peak lung titers in ISVP-infected animals were over 1 log greater than peak lung titers in virion-infected animals. Immunohistochemistry of lung samples taken at 24 hpi from both virion- and ISVP-infected animals revealed type 1 alveolar pneumocytes and monocytes expressing the μNS reovirus nonstructural protein (evidence of viral replication), although we observed many more μNS-positive cells in the lungs of ISVP-infected animals (Fig. 2B). These data suggest that, while endogenous respiratory proteases can productively uncoat reovirus virions *in vitro*, they do not do so efficiently in the murine respiratory tract.

To determine how the kinetics of replication in the respiratory tract impact dissemination, we analyzed viral titers in spleen tissue after intranasal infection with virions or ISVPs (Fig. 2C). Virus was detected as early as 12 hpi in spleen samples from the ISVP-infected animals. At 24 hpi, virion-infected animals began to show evidence of viral spread; by 36 hpi, there was no significant difference between the samples. We considered the possibility that early spread in the ISVP-infected animals reflected quicker drainage of the inoculum, but the virus recovered in the spleen samples was E-64 sensitive, arguing that it represented progeny virions (data not shown). Because mice infected intranasally can swallow some of the inoculum, we measured virus in the intestines at early time points. Recovered titers ranged broadly between animals (data not shown). While this virus might have contributed to the observed spread in our intranasally infected animals, Kauffman and colleagues detected less than half a log of spread to the spleen 24 h after direct intragastric inoculation of 10^8 Lang virions into mice that were similar in age (40). Together, these data suggest that efficient replication of ISVPs in the lung leads to quicker dissemination.

While studies in the murine enteric tract have shown that reovirus virions are converted to ISVPs within minutes after oral inoculation (13), our data suggest that the endogenous respiratory proteases in the lung do not rapidly convert virions to ISVPs. However, the balance of proteases and protease inhibitors in the respiratory tract can be impacted by inflammation (2, 24, 50, 52, 53, 61). Inflammatory cells, recruited to sites of infection or pathology, release a variety of microbicidal products (23, 26, 45). Serine proteases are expressed as components of this response (31), and we have previously shown that one such protease, neutrophil elastase, can promote reovirus infection in cell culture (36). Using the *in vitro* uncoating assay, we investigated the capacity of two other inflammatory proteases, Cat G (expressed by neutrophils) and mast cell chymase, to uncoat reovirus virions. We found that chymase mediated rapid virion disassembly, with almost all σ3 disappearing after 0.5 h (Fig. 3A). The kinetics of σ3 removal from virions treated with Cat G (Fig. 3B) was not as rapid, but by 5 h, particles had lost 70% of σ3, and the underlying μ1C protein had been cleaved to the characteristic δ fragment. When we used the chymase- and Cat G-SVPs in single-cycle growth experiments in E-64-treated L929 cells, we found that they were infectious in the absence of cysteine protease activity (Fig. 3C). The finding that HAT- and chymase-SVPs with uncleaved μ1C replicate to high yields in E-64-treated cells supports other published work suggesting that cleavage of μ1C to yield the δ and φ fragments is not essential for reovirus infection in cell culture (19).

Since our results demonstrated that the inflammatory proteases elastase (36), Cat G, and chymase mediated productive reovirus uncoating, we hypothesized that lung injury or induction of inflammation would potentiate reovirus infection in the lungs by promoting virion uncoating. To test this, we pretreated mice intranasally with lipopolysaccharide (LPS) or UV-inactivated reovirus type 3 Dearing for 24 h to induce inflammation (30, 42, 43, 54). Treated and control mice were then inoculated with type 1 Lang reovirus virions, and virus was quantified from lung tissue 24 h after infection. We recovered significantly more virus at 24 hpi from animals pretreated with UV-inactivated virus and LPS than we did from untreated animals (Fig. 3D). To determine whether preexisting inflammation promoted extracellular conversion of virions to ISVPs, we harvested bronchoalveolar lavage fluid (BALF) samples from untreated or pretreated and infected animals and used these as inocula in single-cycle-yield experiments in E64-treated and untreated L929 cells. In mice that had been pretreated with LPS or UV-inactivated virus, the BALF samples contained significantly more E-64-resistant (uncoated) virus (Fig. 3E), consistent with our hypothesis. To determine the extent to which the pretreatments caused infiltration of inflammatory cells that might also serve as target cells, we quantified the influx of neutrophils and monocytes by the use of Cytospin preparations of the BALF collected 8 h after treatment (Fig. 3F). Pretreatment with LPS resulted in the greatest increase in neutrophil numbers; significant numbers of monocytes were recruited to the lungs after treatment with either LPS or UV-inactivated virus. It was notable that the control treatment (saline solution) also resulted in an influx of inflammatory cells relative to untreated animals; however, levels were significantly lower than in mice pretreated with LPS or UV-treated virus, and they did not correlate with a significant increase in ISVPs in the BALF. Together, our findings reveal that preexisting inflammation in the lungs promotes reovirus uncoating in the respiratory lumen and results in the infiltration of potential target cells. These factors likely contribute to the increases in viral load in mice with preexisting inflammation.

In this report, we have shown that TTSPs can uncoat reovirus virions and promote infection in cell culture. Whether reovirus uncoating in HAT- or TMPRSS2-transfected cells occurs extracellularly, at the cell membrane, or intracellularly remains unknown. Böttcher-Friebertshäuser et al. demonstrated that influenza HA cleavage occurs at the cell membrane in HAT-expressing MDCK cells and in intracellular compartments in TMPRSS2-expressing cells (17). Interestingly, TMPRSS2 overexpression has been associated with prostate cancer (60). Since protease-mediated uncoating is a major determinant of reovirus oncolysis (1), our results suggest that reovirus might selectively target prostate tumors that overexpress and secrete TMPRSS2.

In enteric reovirus infections, pancreatic proteases in the intestinal lumen rapidly convert virions to ISVPs (8). In contrast, in healthy lungs, there are few active proteases in the extracellular environment of the respiratory tract and this may impact reovirus infection. Consistent with this, we found that inoculation with uncoated particles resulted in quicker viral replication in the lungs and spread from the respiratory tract. We explored the capacity of inflammatory serine proteases to promote infection and found that, in addition to neutrophil elastase (36), Cat G and chymase were capable of mediating productive reovirus disassembly *in vitro*.

To probe the role of specific proteases *in vivo*, we attempted to inhibit protease activity in the respiratory tract by prior intranasal

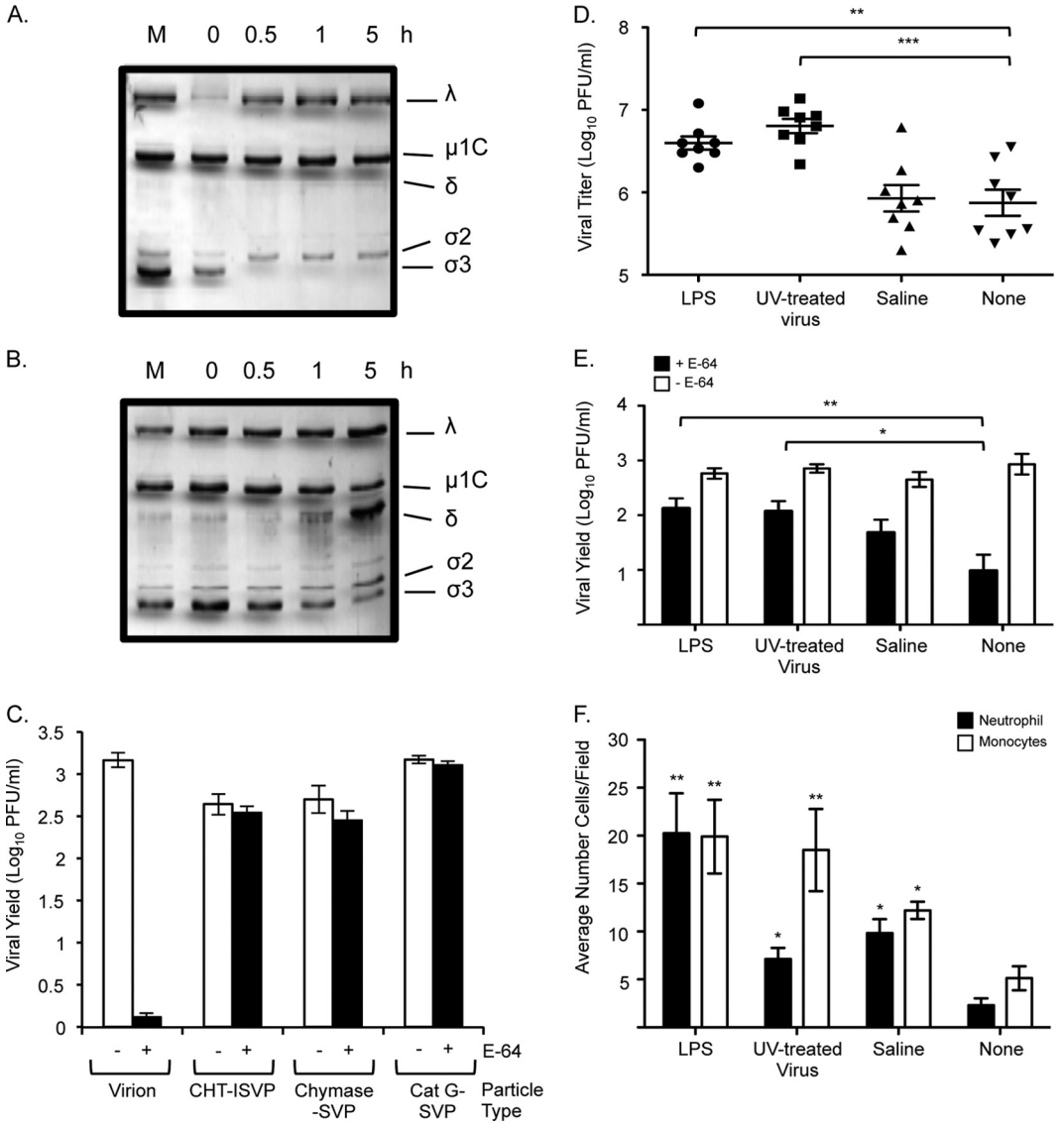

FIG 3 Analysis of the effects of inflammatory proteases and inflammation on reovirus virions and pathogenesis in the murine respiratory model. (A, B, and C) Reovirus strain Lang virions (5×10^{10}) were incubated with purified chymase (Calbiochem) (50 μg/ml) (A) or purified Cat G (Calbiochem) (25 μg/ml) (B) in virion dialysis buffer for the indicated times. Reactions were stopped with 5 mM benzamidine. The mock sample (M) consisted of virions held in reaction buffer in the absence of protease for 5 h. Treated particles were analyzed using SDS–12% polyacrylamide gels, and proteins were visualized by Coomassie staining. The positions of reovirus capsid proteins are labeled. (C) L929 cells were left untreated (white bars) or pretreated for 3 h with 300 μM E-64 (black bars) and infected with Lang virions, CHT-ISVPs, Cat G-SVPs, or chymase-SVPs at an MOI of 5 PFU/cell. Infections were carried out in the presence or absence of E-64 and terminated at 1 day postinfection. Viral yields were determined by plaque assay on L929 cells. Values represent the means ($\pm$ SE) of the results of experiments performed with triplicate samples. (D) Four-week-old CBA/J mice were left untreated or treated intranasally with 1.5 μg of LPS, 1.7×10^9 UV-inactivated T3D virions, or saline solution 24 h prior to intranasal infection with 1×10^7 PFU of reovirus strain Lang virions. At day 1 postinfection, lungs were harvested and viral titers determined by plaque assay on L929 cells. (E and F) Four-week-old CBA/J mice were left untreated or treated and infected as described for panel D. At 8 hpi, BALF was harvested from infected mice. (E) Virus was released from half of the BALF sample by three cycles of freezing and thawing. The presence of uncoated particles and the relative levels of virus were assessed by using the BALF in single-cycle growth experiments. L929 cells were left untreated or pretreated with E-64 for 3 h and then infected with 100 μl of BALF. Viral yields were determined by plaque assay on L929 cells 1 day postinfection. (F) Neutrophils and monocytes in the BALF were quantified by Cytospin analysis and hematoxylin and eosin staining. Error bars indicate SEM. *, $P < 0.05$; **, $P < 0.01$ (as determined by the Mann Whitney test).

inoculation of protease inhibitors. These preliminary studies suggested that the pretreatment protocol itself induced some gross inflammation and slightly increased viral titers from the lung (data not shown). When we modified the model of respiratory infection to specifically induce inflammation prior to reovirus infection (by inoculation with LPS or UV-treated virus), we recovered a higher percentage of ISVPs in the BALF, as well as a significant infiltration of monocytes. These animals also had significantly higher viral yields in the lungs. In contrast, prior inflammation did not promote infection when animals were inoculated with ISVPs (data not shown). These results are consistent with a model in which inflammation promotes reovirus infection

through multiple mechanisms, including increased access to target cells and the presence of inflammatory proteases. Our findings may have broader implications for chronic pulmonary disease. Inflammation induced under a variety of chronic conditions, including asthma, emphysema, chronic obstructive pulmonary disease, and acute respiratory distress syndrome (39, 41), may further compromise human health by providing an environment that is conducive to viral infection. Further work is needed to address the possibility that other respiratory viruses (including influenza virus and SARS coronavirus) can also be activated by inflammatory proteases.

ACKNOWLEDGMENTS

We thank Linse Lahti, Megan Ahl Fischer, Jesse Bahe, and Lindsey Foran for excellent technical support and Stephen Schmechel for analysis of histopathology. We also thank Max Nibert and Terry Dermody for their generous gifts of μNS and σNS antiserum. We are also grateful to Mikhail Matrosovich for providing the HAT and TMPRSS2 plasmids and Stephen Rice for providing the pcDNA22 plasmid. Stephen Rice, Linse Lahti, and Wade Schulz provided helpful feedback on the manuscript.

This work was supported by Public Health Service grant AI-045990 from the National Institutes of Health.

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