Type-I interferons induce lung protease responses following respiratory syncytial virus infection via RIG-I-like receptors

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The role of proteases in viral infection of the lung is poorly understood. Thus, we examined matrix metalloproteinases (MMPs) and cathepsin proteases in respiratory syncytial virus (RSV)-infected mouse lungs. RSV-induced gene expression for MMPs -2, -3, -7, -8, -9, -10, -12, -13, -14, -16, -17, -19, -20, -25, -27, and -28 and cathepsins B, C, E, G, H, K, L1, S, W, and Z in the airways of Friend leukemia virus B sensitive strain mice. Increased proteases were present in the bronchoalveolar lavage fluid (BALF) and lung tissue during infection. Mitochondrial antiviral-signaling protein (MAVS) and TIR-domain-containing adapter-inducing interferon-β-deficient mice were exposed to RSV. *Mavs*-deficient mice had significantly lower expression of airway MMP-2, -3, -7, -8, -9, -10, -12, -13, and -28 and cathepsins C, G, K, S, W, and Z. In lung epithelial cells, retinoic acid-inducible gene-1 (RIG-I) was identified as the major RIG-I-like receptor required for RSV-induced protease expression via MAVS. Overexpression of RIG-I or treatment with interferon-β in these cells induced MMP and cathepsin gene and protein expression. The significance of RIG-1 protease induction was demonstrated by the fact that inhibiting proteases with batimastat, E64 or ribavirin prevented airway hyperresponsiveness and enhanced viral clearance in RSV-infected mice.

INTRODUCTION

Microbial infections in the airways contribute to tissue remodeling and are also a major factor in exacerbating underlying diseases, which have been associated with cytokine and chemokine release from resident lung cells.[1] A protease/antiprotease imbalance can equally be associated with inflammation and extracellular matrix degradation.[2] Proteases have a number of functional effects that contribute to tissue destruction including tissue remodeling, cleavage of soluble innate factors, and activation of receptors. Alternatively, proteases also have a critical role in microbial clearance as neutrophil proteases are critical for microbial killing.[3] However, some proteases may even facilitate viral entry and replication into human cells.[4] Thus, determining how viral infection impacts on protease expression, release, and activity and the subsequent protease effects on immune responses and lung microenvironment remain important areas to explore.

Matrix metalloproteinases (MMPs) and cathepsins are clinically important proteases that are elevated in many diseases. MMPs are produced by a wide variety of cell-types, including epithelium, fibroblasts, neutrophils, and macrophages.[5] MMPs act on a variety of non-matrix extracellular proteins, such as cytokines, chemokines, receptors, junctional proteins, and antimicrobial peptides, to mediate a broad range of biological processes, such as repair, immunity, and angiogenesis.[5] Similarly, cathepsins are produced by several cell-types and tissues and are known to undergo activation by cytokines.[6] Respiratory syncytial virus (RSV) is a virus frequently reported in infants, the elderly, immunocompromised patients as well as healthy adults.[7] Infection alters protease profiles, with MMP-3 and -10 expression being sensitive to RSV inoculation in nasal epithelial cells[8] and MMP-3 levels in nasopharyngeal aspirates are associated with RSV disease severity.[9] RSV induces MMP-9 gene expression in human bronchial epithelial cells[10] and MMP-9 and -2 were

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activated by RSV in BALB/c mouse.[11] Bovine RSV can act synergistically with *Histophilus somni* supernatant to elevate MMP-1 and -3 levels, leading to enhanced collagen breakdown and facilitating viral infection efficiency in bovine alveolar type-2 cells.[12] Therefore, previous studies show that RSV induces several proteases and suggest that RSV-inducible proteases may have a major role in disease progression. Proteases become expressed in response to microbial product stimuli[13] with pathogen recognition receptors having a major role in protease gene regulation when utilizing microbial mimicking agonists.[14,15] Pathogen recognition receptors, such as toll-like receptors (TLR) and retinoic acid-inducible gene-1 (RIG-I)-like receptors (RLRs), induce major signaling cascades in response to viral stimulation.[16] Both TLR-mediated TIR-domain-containing adapter-inducing interferon-β (Trif) signaling and RLR can modulate similar immune processes to regulate cytokine production.[17,18] The viral load of RSV correlates with the messenger RNA levels of the RLR, RIG-I.[19] RIG-I and melanoma differentiation-associated protein 5 (MDA5) activate the mitochondrial antiviral-signaling protein (MAVS) to trigger an antiviral response.[20] However, little is known about the role of pathogen recognition receptors in RSV-induced protease expression; although proteases have been shown to modulate TLR3 and RIG-I signaling[21] and inhibition of MMP-9 activity in bronchial epithelial cells prevents syncytia formation and blocks RSV multiplication.[10] Therefore, profiling the protease response during RSV infection and characterizing their regulation and role in disease progression may be beneficial for future treatment of RSV infection.

In this study, we investigate MMP and cathepsin expression responses to RSV infection. *In vivo* and *in vitro* approaches were utilized to identify the major regulatory signaling pathways in RSV-induced protease expression. The influence of Trif and MAVS signaling pathways were examined on RSV-induced protease expression, with RLR-dependent MAVS signaling observed to have a major role in RSV-induced MMP and cathepsin expression. These findings indicate that viral infections significantly enhance host protease responses, in a type-I interferon-dependent mechanism. Furthermore, we show that the RLR pathways are key players in the host protease response to viral infection and inhibition of proteases may be beneficial in clearing RSV from the airways.

RESULTS

RSV infection induces MMP and cathepsin expression and activity

Increased protease levels have been frequently observed in human airway diseases[22] and have a critical role in microbial killing.[3] While it is established that a viral-induced host proteases response occurs, when and which proteases are induced in RSV-infected lungs is not yet elucidated. Here we show that mice exposed to RSV infection have increased airway collagenase and elastase activity in their bronchoalveolar lavage fluid (BALF) (**Figure 1a**). Elastase activity was observed as early as 24 h post infection. Both elastase and collagenase activity persisted beyond 9 days post RSV challenge. Protease activity mimicked the RSV N copy number and viral titer within the lung tissue, with reduced protease activity observed upon RSV clearance (**Figure 1b**). RSV-infected mice also lost weight during infection (**Figure 1c**) and had increased BALF immune cell infiltration (**Figure 1d**). Not surprisingly, RSV infection resulted in an infiltration of macrophages, neutrophils, and lymphocytes into the lung (**Figure 1d,e**).

The influence of viral infection on protease expression was investigated in greater detail via quantitative PCR (qPCR) by examining the MMP and cathepsin families of proteases. Mice infected with RSV have significant gene expression increases for MMP-2, -3, -7, -8, -9, -10, -12, -13, -14, -16, -17, -19, -20, -25, -27, and -28 (**Figure 2a–h**). Additionally, cathepsins B, C, E, G, H, K, L1, S, W, and Z were all enhanced by RSV infection (**Figure 2i–m**). Of note, 9 days post RSV lung infection MMPs -2, -8, -9, -10, -12, -13, -14, -16, -19, -25, -27, -28 and cathepsins E, G, K, L1, S, W, and Z remained significantly increased in RSV-infected lungs (**Figure 2**). Protein analysis was performed to confirm qPCR data. As illustrated in **Figure 3**, RSV infection enhanced BALF and lung tissue MMP and cathepsin protein levels and activity. MMP-2, -3, -8, -9, and -12 BALF protein levels were quantified by multiplex analysis (**Figure 3a**). RSV infection increased BALF gelatinase activity, which demonstrated an early MMP-9 response and a later MMP-2 response to RSV challenge (**Figure 3b**). Western blot on lung tissue confirmed that RSV exposure increased cathepsin E, S, G, K, B, W, and Z levels 7 days post infection (**Figure 3c**). Cathepsin B, G, and S activity levels were elevated in BALF from RSV-infected mice (**Figure 3d**).

RSV induces the MAVS/MDA5/RIG-I/TLR3 pathways in mouse lungs

RSV increased protein levels of MDA5, RIG-I, MAVS, MyD88, and Trif (**Figure 4a**). Additionally, phosphorylation of intereferon beta (IFN-β) regulatory factor 3 and TANK-binding kinase 1 were observed following RSV infection (**Figure 4a**). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 activity were induced by RSV 24 h post challenge and continued to persist beyond 9 days (**Figure 4b**). As the TLR3/4 and RLR pathways were altered by RSV,[19] *Trif* and *Mavs* knockout (KO) mice were used to examine the importance of these pathways on protease production *in vivo*. *Mavs* KO mice were utilized as MAVS links RIG-I and MDA5 to antiviral effectors responses. *Trif* KO mice were chosen as both TLR3 and TLR4 are activated by RSV and are the only TLRs that utilize Trif signaling. Mice were exposed to RSV and killed every second day until 9 days post RSV challenge. Gene expression studies were performed on days post RSV challenge that corresponded with the maximal level of gene expression for each protease as determined in **Figure 2**. Loss of *Mavs* expression prevented RSV-induced MMP-2, -3, -7, -8, -9, -10, -12, -13, and -28 and cathepsins C, G, K, S, W, and Z gene expression (**Figure 5**). Loss of *Trif* expression altered the RSV-induced gene expression of MMP-8, -28 and cathepsins G, K, L1, S and Z (**Figure 5**).

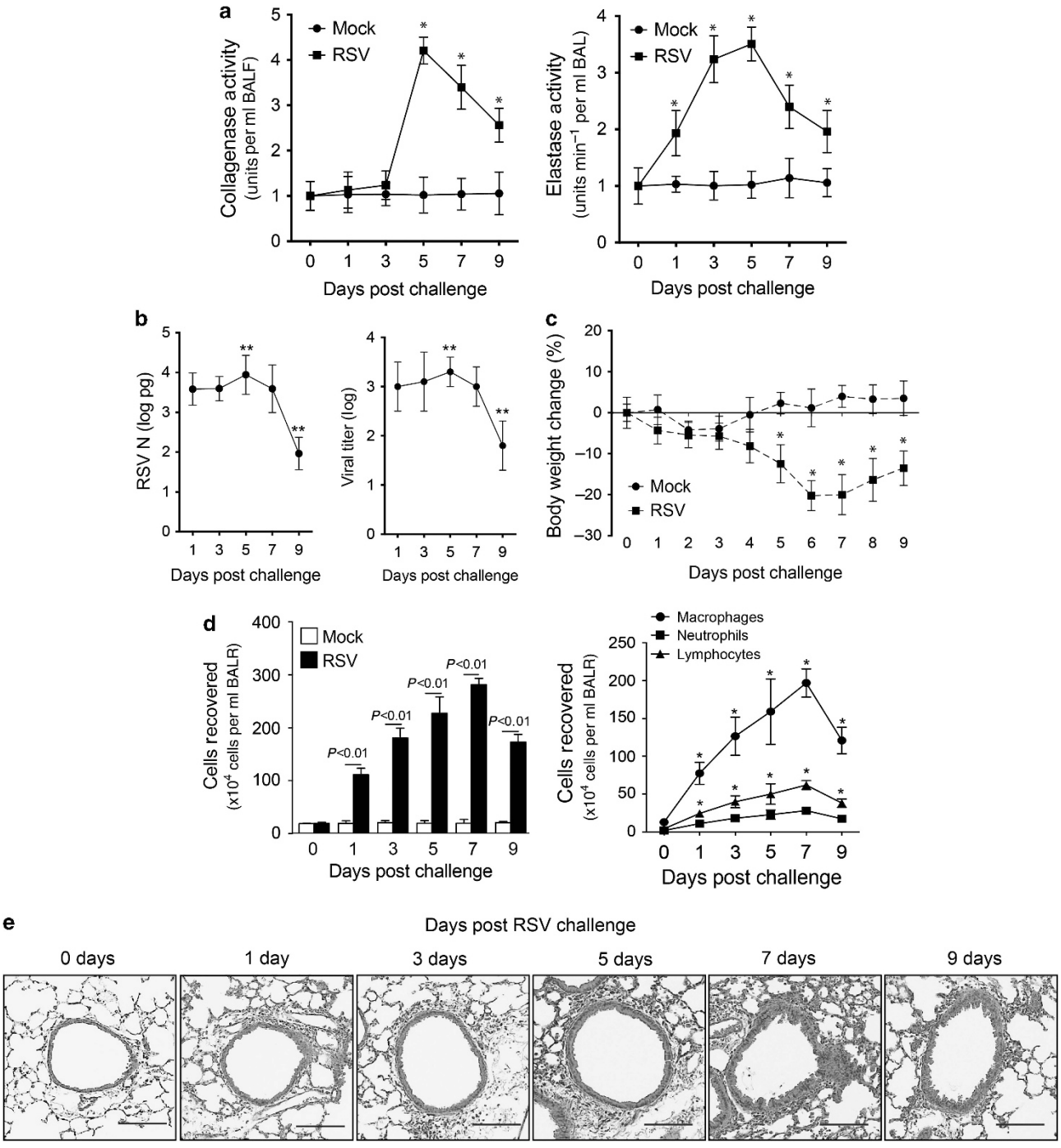


Figure 1 Respiratory syncytial virus (RSV) infection induces protease activity in the airways. Friend leukemia virus B sensitive strain mice were infected with 1×10^6 plaque forming units of RSV and a group of animals were killed at day 0, 1, 3, 5, 7, and 9 post infection. (**a**) Bronchoalveolar lavage fluid (BALF) had greater collagenase and elastase activity in animals infected with RSV compared with mock controls. (**b**) RSV N copy number and viral load were greatest 5 days post RSV challenge and were at the lower level of detection by day 9. (**c**) RSV infection resulted in a significant drop in body weight in animals and (**d**) an increase in BALF cellularity. Graphs are represented as mean ± s.e.m., where each measurement was performed three times on 12 animals per group. * and ** represents a *P*-value < 0.05 compared with mock-treated mice or day 1 RSV-treated mice, respectively. *P*-values shown comparing RSV to mock-treated mice are connected by a line. (**e**) Representative images of mice lungs from each RSV-treated group (bar = 10 μM).

Interestingly, *Trif* expression suppressed cathepsin G gene expression (**Figure 5**). Tissue and BALF protein analysis confirm *Trif* or *Mavs* regulation of protease expression (**Figure 6a,b**). Loss of *Mavs* impacts on tissue levels of cathepsin E, G, K, S, W, and Z (**Figure 6a**) and BALF levels of MMP-2, -3, -8, -9, and -12 (**Figure 6b**). Loss of *Trif* impacted on cathepsin S tissue levels (**Figure 6a**). In addition, *Trif* and *Mavs* KO mice displayed a similar loss in body weight following RSV infection as wild-type mice (**Figure 6c**), but loss of *Mavs* or *Trif* resulted in a greater viral load in the airway

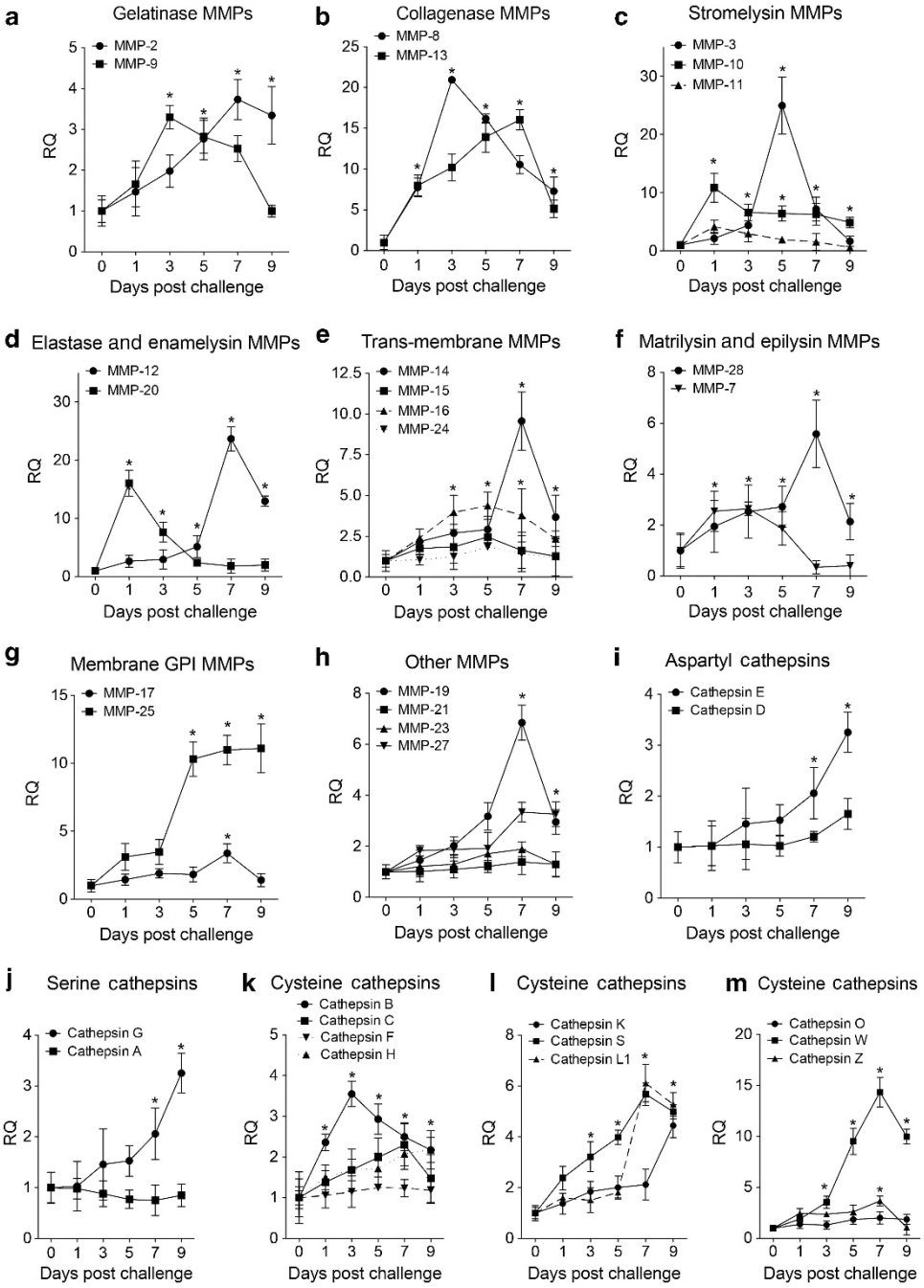


Figure 2 Respiratory syncytial virus (RSV) infection induces matrix metalloproteinase (MMP) and cathepsin gene expression. Friend leukemia virus B sensitive strain mice were infected with 1×10^6 plaque forming units of RSV and animals were killed every second day as previously described. (**a**–**h**) MMP and (**i**–**m**) cathepsin lung gene expression were analyzed by quantitative PCR. Graphs are represented as relative quantification of the mean ± s.e.m., with each measurement performed three times on 12 animals per group. *Represents a P-value < 0.05 compared with mock-treated mice on each corresponding day.

tissue on day 9 following infection compared with wild-type mice (**Figure 6d**).

Type-I interferon responses regulate RSV-induced protease expression in human small airway epithelial (SAE) cells

RSV is known to readily infect the airway epithelium[23] and is detectible in the airways using immunofluorescence (**Figure 7a**). Therefore to confirm our *in vivo* findings and identify which RLR is regulating MAVS-induced protease production, human airway epithelial cells were infected with RSV and protease expression profiles were analyzed by qPCR (**Figure 7**). Gene expression for MMP-1, -3, -7, -8, -9, -10, 12, and 13 were induced in SAE cells upon RSV infection (**Figure 7b**) but were not observed when RSV was ultraviolet-treated (**Supplementary Figure S1A** online). Multiplex

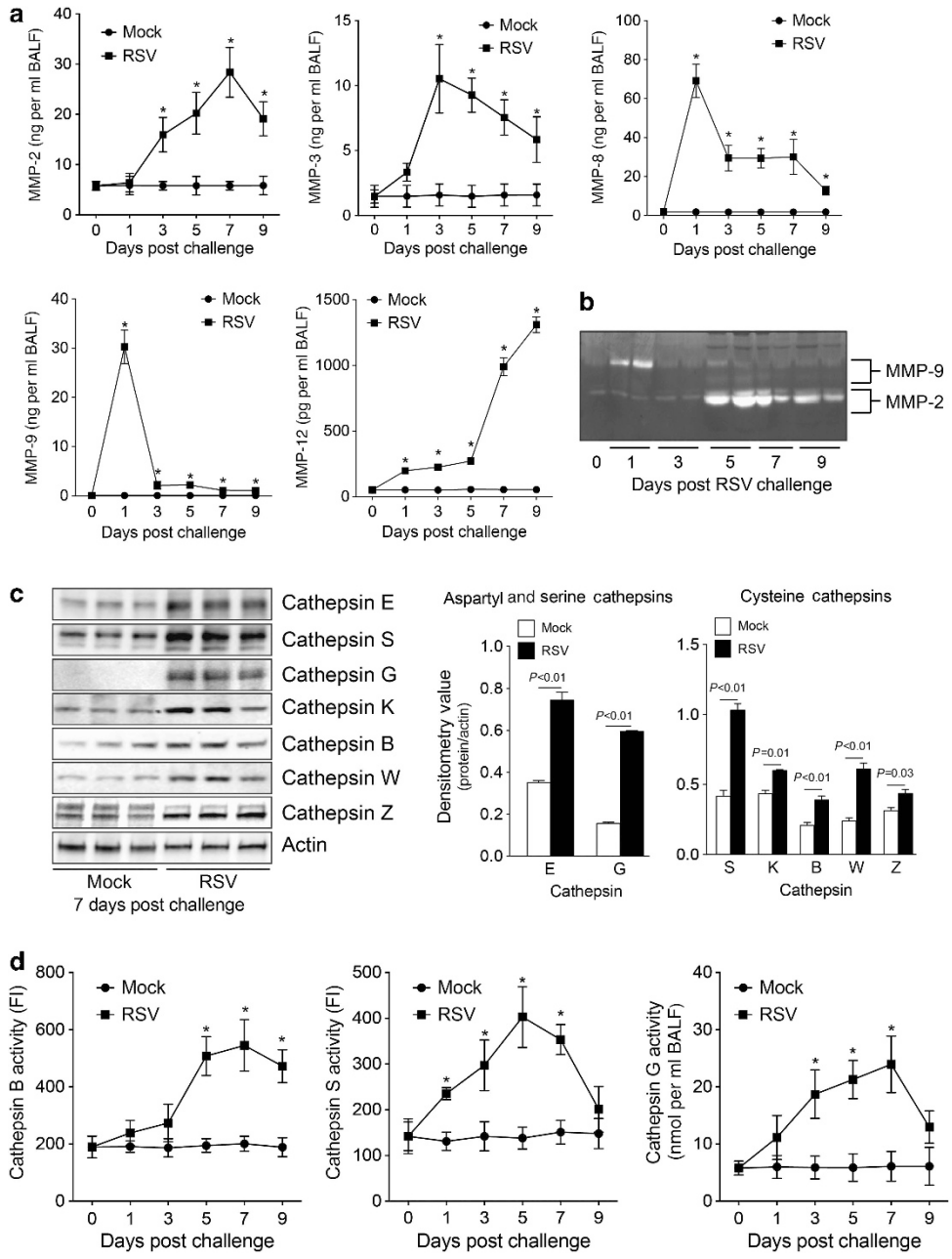


Figure 3 Respiratory syncytial virus (RSV) infections induce matrix metalloproteinases (MMPs) and cathepsin protein expression and activity. (**a**) Bronchoalveolar lavage fluid (BALF) MMP-2, -3, -8, -9 and -12 levels were determined by multiplex analysis on mock-treated and RSV-treated animals. (**b**) Gelatinase activity was analyzed in BALF using gelatin zymography. Bands corresponding to MMP-2 and MMP-9 are highlighted. (**c**) Cathepsin E, S, G, K, B, W, and Z lung protein levels were analyzed by immunoblotting and corresponding densitometry analysis. (**d**) Cathepsin B, S, and G activity levels were determined in BALF. FI represents fluorescent intensity. Graphs are represented as mean ± s.e.m, where each measurement was performed three times on 12 animals per group. *Represents a P-value < 0.05 compared with mock-treated mice. P-values shown comparing both treatments are connected by a line.

analysis demonstrated that increased MMP-1, -3, -7, -8, -9, -10, and -13 were observed in the media of RSV-infected cells (**Figure 7c**). Only cathepsins E and G were induced in SAE cells upon RSV infection (**Figure 7d** and **Supplementary Figure S1B**). Interestingly, using an immunofluorescence approach, only cells positive for RSV antigen had increased MMP-9 and cathepsin E (**Figure 7e**). The tissue inhibitors of metalloproteinases-1, -2, and -3 were unaltered by RSV stimuli in SAE cells, as observed in the media from SAE cells (**Supplementary Figure S2**).

Since RSV induced a wide range of the proteases in a similar manner to that observed *in vivo*, SAE cells were transfected with silencing RNA (siRNA) for Trif, MDA5, RIG-I, laboratory of genetics and physiology 2 (LGP2), and MAVS (see

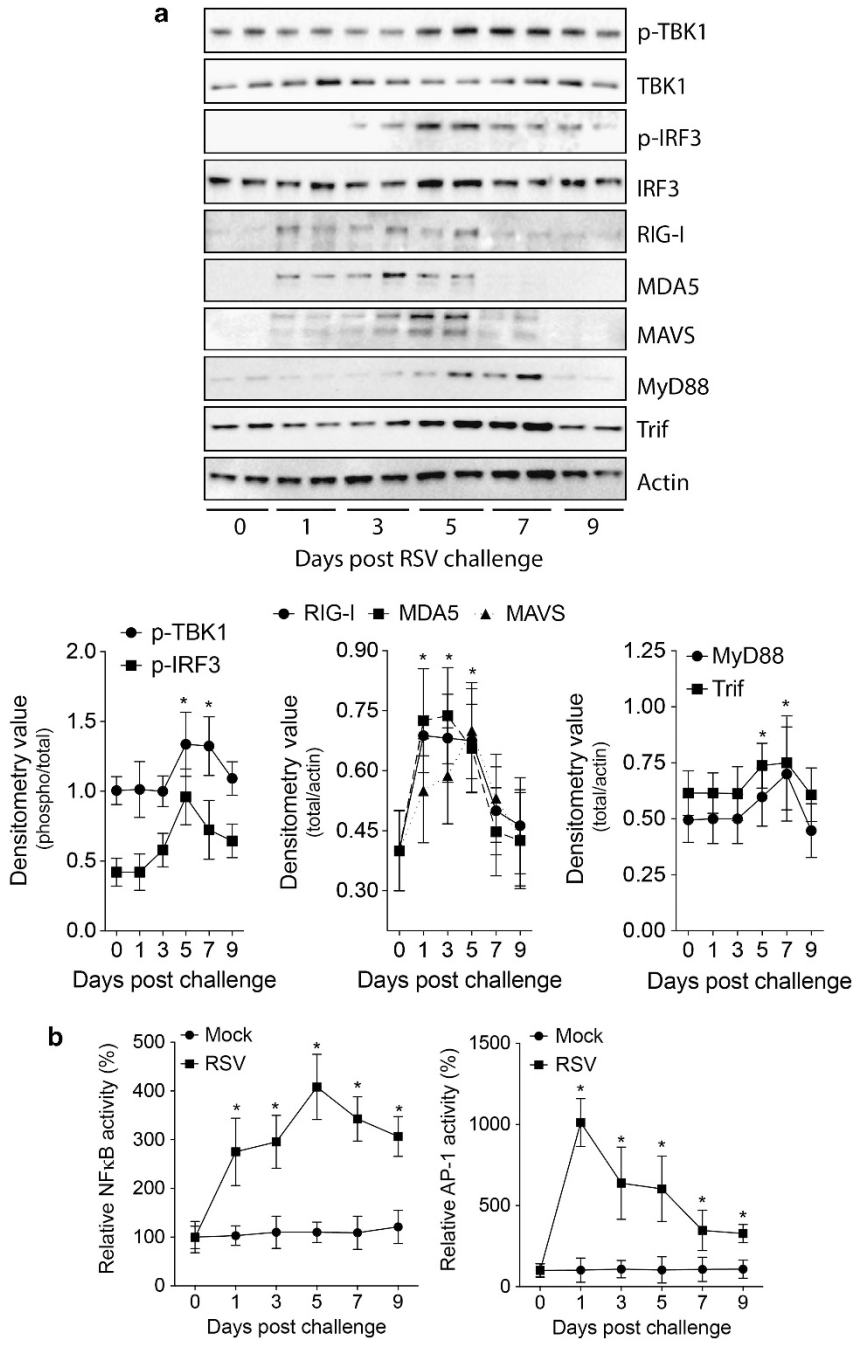


Figure 4 Respiratory syncytial virus (RSV) induces the mitochondrial antiviral-signaling protein (MAVS)/melanoma differentiation-associated protein 5 (MDA5)/retinoic acid-inducible gene-1 (RIG-I)/toll-like receptor 3 (TLR3) pathways in mouse lungs. (**a**) Immunblots were performed to examine levels of MDA, RIG-I, MAVS, MyD88 and TIR-domain-containing adapter-inducing interferon-β (Trif) protein and interferon regulatory factor 3 (IRF3) and TANK-binding kinase 1 (TBK1) phosphorylation in lung tissue of RSV-infected animals. Densitometry analysis was performed against actin or total protein (TBK1 and IRF3) levels for all samples from multiple immunoblots. (**b**) Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) nuclear activity levels were determined in the lung tissue of mock- and RSV-treated mice. Graphs are represented as mean ± s.e.m., where each measurement performed three times on 12 animals per group. *Represents a *P*-value < 0.05 compared with mock-treated mice on each corresponding day.

Supplementary Figure S3 for siRNA transfection efficiency) to confirm the *in vivo* data and also to determine the RLR responsible for this protease induction. Several of the siRNAs altered protease expression, however RIG-I and MAVS expression were critical for the majority of RSV-induced proteases (**Table 1**). This is of interest, as RSV viral load correlates with RIG-I messenger RNA levels in a study of bronchiolitis in infants.[19] In fact, overexpression of RIG-I in SAE cells upregulated the same proteases as RSV stimulation (**Figure 8**). Therefore, these results show

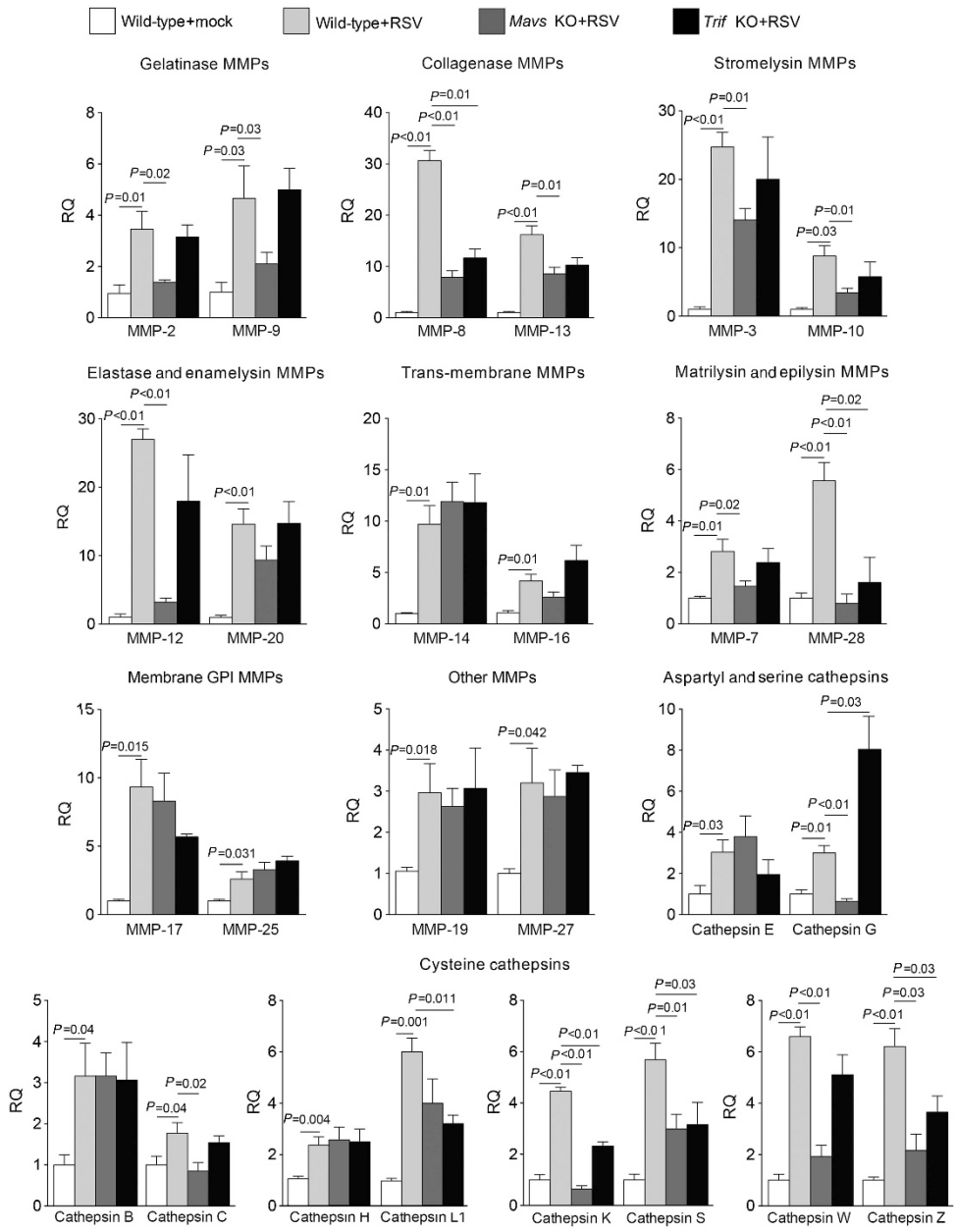


Figure 5 Mitochondrial antiviral-signaling protein (MAVS) is required for the gene expression of the majority of respiratory syncytial virus (RSV)- inducible airway proteases. Wildtype, TIR-domain-containing adapter-inducing interferon-β (*Trif*) (*Ticam1*) and *Mavs* knockout (KO) mice were infected with 1×10^6 plaque forming units of RSV and animals were killed every second day as previously described. Matrix metalloproteinases (MMP) and cathepsin gene expression in lung tissue was analyzed by quantitative PCR on the day post infection with the maximal level of gene expression for each protease, as determined in **Figure 2**. MMP-10 and -20 were examined 1 day post infection. MMP-7, -8, -9 and cathepsin B were examined 3 days post infection. MMP-3, -16 and -17 were examined 5 days post infection. MMP-2, -12, -13, -14, -19, -25, -27, -28, cathepsin C, L1, S, W, and Z were examined 7 days post infection. Cathepsin E, G, H, and K were examined 9 days post infection. Results are represented as relative quantification (RQ) of the mean ± s.e.m. when compared with mock-treated animals on each corresponding day, with each measurement performed three times on 12 animals per group. *P*-values shown comparing treatments are connected by a line.

that RLRs, such as RIG-I, have a major role in RSV-mediated protease expression.

As RIG-I signaling regulates RSV-induced protease expression, type-I interferon stimulus was examined for induction of protease expression. SAE cells treated with 1,000 units of recombinant human IFN-β lead to gene expression induction of the same proteases seen in RSV stimulation (**Figure 8c,d**). Equally, silencing the IFN-α/β receptors (IFNAR1 and IFNAR2) moderately subdued RSV from inducing gene expression of MMPs and cathepsins (**Figure 8e**). Therefore RSV-induced protease expressions are in part an indirect effect of elaborated cytokines, such as a type-I interferon response.

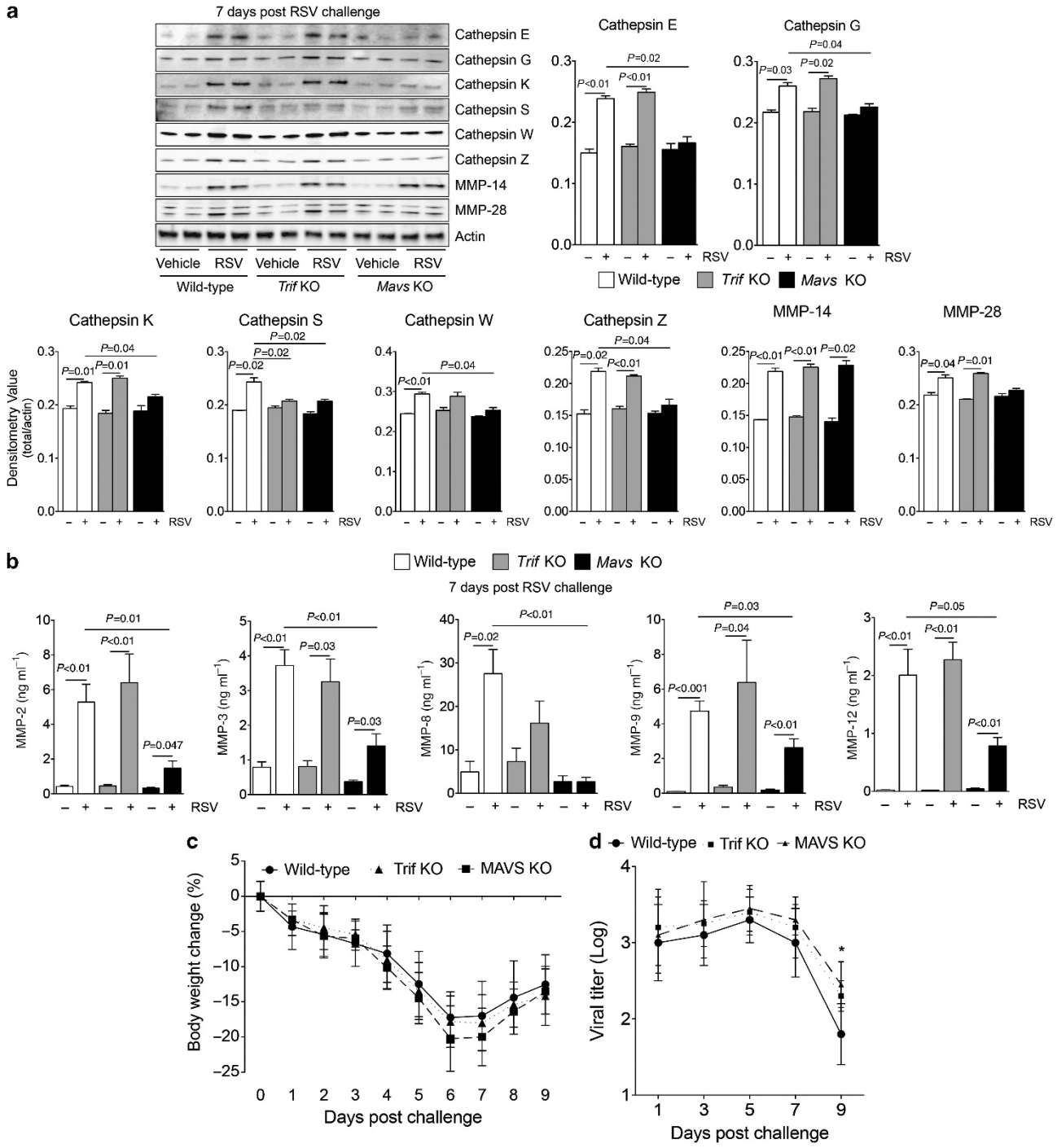


Figure 6 Mitochondrial antiviral-signaling protein (MAVS) is required for the protein expression of the majority of respiratory syncytial virus (RSV)- inducible airway proteases. (**a**) Immunoblots (Cathepsin E, G, K, S, W, Z, matrix metalloproteinases (MMPs)-14, MMP-28 and actin) were performed on lung tissue 7 days post RSV challenge and densitometry analysis was determined. The Y-axis represents densitometry value for all graphs. (**b**) Multiplex analysis (MMP-2, -3, -8, -9, and -12) was performed on bronchoalveolar lavage fluid (BALF) 7 days post RSV challenge. (**c**) RSV infection resulted in a drop in body weight in all animal groups and (**d**) a significant increase in the viral load 9 days post RSV challenge in the *Mavs* knockout (KO) mouse group compared with mock-treated mice. Graphs are represented as mean ± s.e.m., where each measurement was performed three times on 12 animals per group. *P*-values shown comparing both treatments connected are by a line. *Represents a *P*-value < 0.05 comparing *Mavs* KO mice to wild-type mice 9 days post RSV challenge.

Inhibition of protease activity enhanced RSV clearance from the airways

Protease inhibitors were utilized *in vivo* to assess the functional role of enhanced proteases in the airways during RSV infection. Wild-type animals were administered a MMP inhibitor (batimastat) or cathepsin inhibitor (E64) daily during RSV infection. Batimastat, a potent inhibitor of MMP -1, -2, -3, -7 and -9, and E64, an effective cysteine protease inhibitor, were

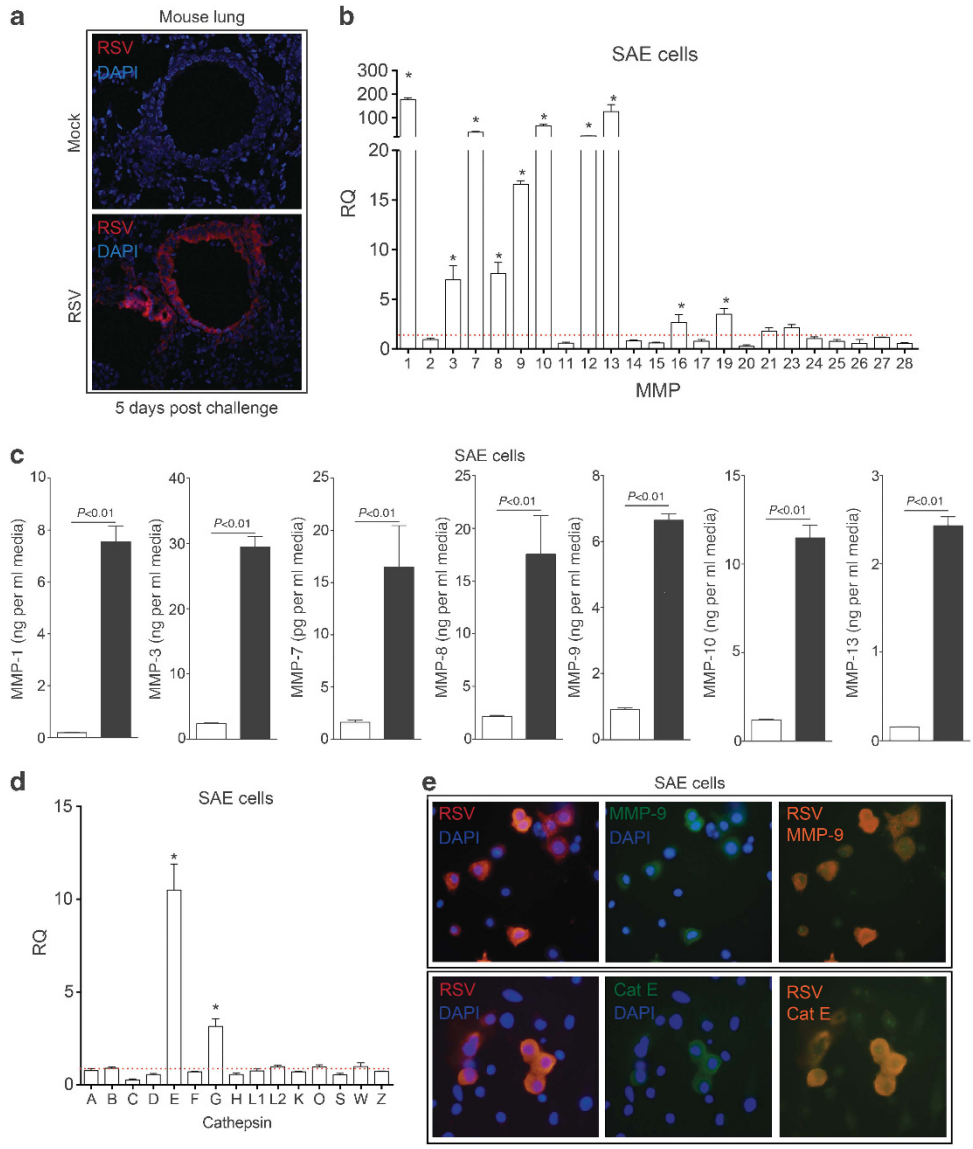


Figure 7 The matrix metalloproteinases (MMPs) and cathepsin gene expression profile in human small airway epithelial (SAE) cells exposed to Respiratory syncytial virus (RSV). (**a**) Immunofluorescence was performed on tissue from mock or RSV-infected mice 5 days post RSV challenge with an antibody that recognizes RSV antigen (red). (**b**) MMP gene expression performed in SAE cells treated for 24 h with mock or RSV was analyzed by quantitative PCR (qPCR). (**c**) MMP-1, -3, -7, -8, -9, -10, and -13 in cell culture supernatants were determined by multiplex analysis following RSV infection. (**d**) Cathepsin gene expression was performed in SAE cells treated for 24 h with mock or RSV was analyzed by qPCR. (**b** and **d**) Dotted line represents mock-treated level of expression. *Represents a *P*-value <0.05 compared with mock-treated mice. (**e**) Co-immunofluorescence was performed on cells for either MMP-9 or cathepsin E (green) and with an antibody that recognizes RSV antigen (red) (bars $=20\,\mu m$). Merged images demonstrate co-localization (orange). Graphs are represented as mean ± s.e.m., where $n=10$ per group. *P*-values shown comparing both treatments are connected by a line.

intraperitoneal administered daily. An antiviral drug, ribavirin, was also administered to a group of animals as ribavirin is administered to patients with severe RSV infection. Also, ribavirin can inhibit MMP-9 activity in THP-1 cells.[24] Animals were killed 7 days post RSV challenge and all measurements were carried out on this day. *In vivo* administration of batimastat and E64 significantly inhibited protease activity in the airways (**Figure 9a,b**). Interestingly, ribavirin treatment prevented protease activity in mouse airways during RSV infection (**Figure 9a,b**). This inhibition was comparable to both the protease inhibitors. Animals receiving a protease inhibitor or ribavirin did not lose weight during the week of infection and demonstrated faster clearance of RSV from the airways (**Figure 9c,d**). Surprisingly, there were reduced immune cells recruited to the airways in animals receiving protease inhibitor or ribavirin (**Figure 9d**). Inhibition of proteases also subdued RSV-induced airway hyperresponsiveness, demonstrated by respiratory system resistance measurements during a methacholine dose challenge (**Figure 9e**). Interleukin (IL)-4 has a major role in allergic response to RSV infection resulting

in the development of airway hyperresponsiveness and lung eosinophilia.[25] Animals receiving a protease inhibitor or ribavirin had reduced IL-4 responses to RSV (**Figure 9f**), which may contribute to the changes in airway hyperresponsiveness to RSV. IFN-β levels were unaltered in all animal groups infected with RSV (**Figure 9f**).

DISCUSSION

Several studies have investigated the induction of proteases by RSV as early as 1999,[26] but the role of these protease families in disease severity and viral clearance has not been established. We characterized how two families of proteases respond to RSV infection and demonstrate that the induction of proteases by RSV is primarily regulated by RLRs, such as RIG-I, in a type-I-dependent manner. Prior to this study, RSV infections were known to induce proteases, such as neutrophil elastase[26] and MMP-9,[11] but little was known about the influence of RSV infection on protease expression and the subsequent impact on the lung. We demonstrated that RSV induces expression of protease networks that contribute to disease progression. This study highlights the impact of RSV infection on the proteolytic response within the airways and the potential to target proteases to treat RSV infection.

Prior to undertaking this study, we believed that RSV-induced proteases were enhanced via a TLR3-/Trif-dependent manner, as polyinosinic–polycytidylic acid, a synthetic analog of double-stranded RNA, enhanced several MMPs[14,15] and TLR3/Trif are significantly induced by RSV. However, TLR3/Trif only partially regulated the protease response and RIG-I exerted a far more substantial effect on airway proteases responses. RLRs largely recognize double-stranded RNA, which is produced during the replication of many viruses.[27] Sendai virus defective interfering RNA[28] and the genomic "panhandle" structure of influenza virus[29] activate RLR signaling. It has been speculated that these "panhandle" structures allow negative-strand RNA viral genomes to achieve partially double-stranded RNA.[30] Exactly how RNA triggers the RLR pathway for most RNA viruses, including RSV, is yet to be identified. In rhinovirus studies, the bronchial epithelium antiviral response initially requires recognition of rhinovirus infection by TLR3/Trif and a subsequent RNA helicases response,[16] suggesting a contribution of both pathways. Indeed,

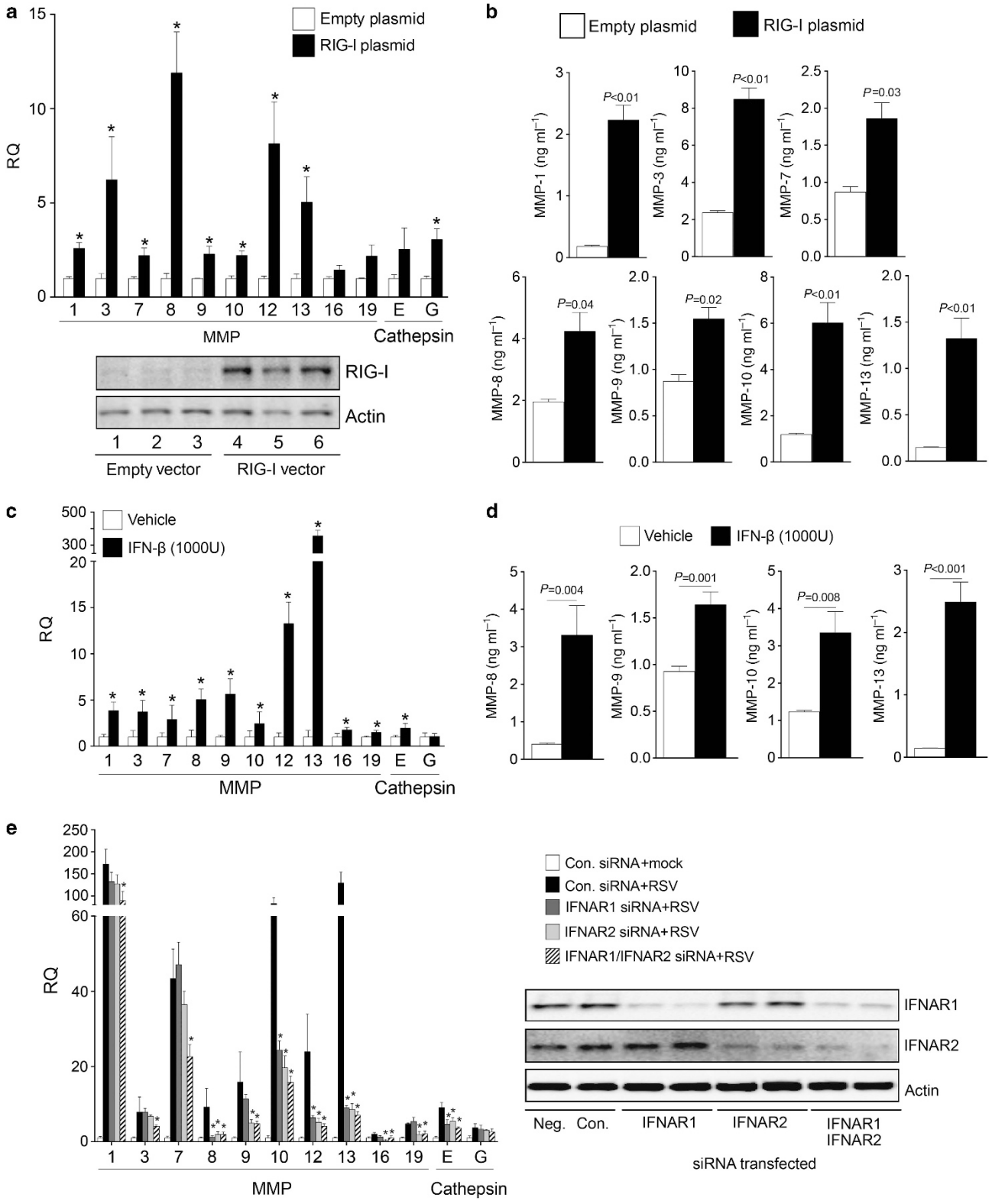
Table 1 RSV-induced MMP and cathepsin expression in human in SAE cells requires RIG-I and MAVS expression

	siRNA						
	Neg. control	Neg. control	Trif	MDA5	RIG-I	LGP2	MAVS
Target	Vehicle	RSV	RSV	RSV	RSV	RSV	RSV
MMP-1	1.0 ± 0.2	172.8 ± 27.2	109.6 ± 23.9	81.8 ± 8.2	**32.4 ± 5.9**	108.6 ± 43.7	**32.6 ± 6.9**
MMP-3	1.0 ± 0.2	7.9 ± 1.2	**3.9 ± 0.1**	**4.1 ± 0.4**	**2.4 ± 0.5**	5.2 ± 0.4	**3.3 ± 1.5**
MMP-7	1.0 ± 0.3	42.8 ± 12.4	33.0 ± 1.7	25.7 ± 1.5	**10.8 ± 2.1**	**11.7 ± 1.5**	**3.8 ± 1.9**
MMP-8	1.0 ± 0.2	9.1 ± 1.7	9.7 ± 2.5	**4.2 ± 0.4**	**2.3 ± 0.3**	10.8 ± 2.7	**4.4 ± 0.6**
MMP-9	1.0 ± 0.2	16.5 ± 2.3	12.2 ± 1.7	**8.8 ± 1.1**	**3.7 ± 0.8**	14.5 ± 1.6	**5.1 ± 0.9**
MMP-10	1.0 ± 0.3	82.9 ± 18.7	95.1 ± 31.1	**32.7 ± 3.0**	**8.8 ± 1.5**	72.9 ± 1.4	**16.9 ± 1.3**
MMP-12	1.0 ± 0.3	23.7 ± 3.4	**10.7 ± 0.9**	**14.0 ± 1.8**	**4.1 ± 0.9**	24.7 ± 4.3	**1.7 ± 1.2**
MMP-13	1.0 ± 0.2	129.1 ± 35.8	91.5 ± 1.8	84.9 ± 7.6	**27.3 ± 3.9**	104.0 ± 9.6	**29.7 ± 7.3**
MMP-16	1.0 ± 0.1	1.9 ± 0.3	**0.9 ± 0.2**	1.3 ± 0.1	**0.5 ± 0.1**	2.2 ± 0.4	2.5 ± 0.5
MMP-19	1.0 ± 0.5	3.9 ± 0.4	4.2 ± 0.8	**1.9 ± 0.3**	**1.0 ± 0.1**	**1.5 ± 0.9**	**0.6 ± 0.2**
Cathepsin E	1.0 ± 0.2	9.1 ± 2.3	6.0 ± 0.3	4.8 ± 0.6	**2.6 ± 0.4**	6.7 ± 0.3	**1.2 ± 0.9**
Cathepsin G	1.0 ± 0.2	3.7 ± 0.8	**12.4 ± 1.8**	2.0 ± 0.2	2.1 ± 0.4	3.2 ± 0.2	2.6 ± 0.3

LGP2, laboratory of genetics and physiology 2; MAVS, mitochondrial antiviral-signaling protein; MMP, matrix metalloproteinase; Neg., negative; RIG-I, retinoic acid-inducible gene-1; RSV, respiratory syncytial virus; SAE, small airway epithelial; SiRNA, silencing RNA; Trif, TIR-domain-containing adapter-inducing interferon-β.
Values are represented as mean ± s.e.m., where $n = 10$ per group. Bold numbers represent a P-value 0.05 compared with RSV-treated negative control siRNA transfected SAE cells.

Figure 8 Overexpression of retinoic acid-inducible gene-1 (RIG-I) in human small airway epithelial (SAE) cells and intereferon beta (IFN-β) stimulation induces protease expression. (**a**) matrix metalloproteinases (MMPs) and cathepsin gene expression was performed on SAE cells transfected with either an empty vector (pCMV6-Entry) or with the same vector overexpressing *RIG-I* (*DDX58*). Immunoblots were performed to determine RIG-I protein levels compared with actin and three individual transfections are presented. (**b**) MMP-1, -3, -7, -8, -9, -10, and -13 media levels were determined by multiplex analysis 24 h following respiratory syncytial virus (RSV) infection. (**c**) MMP and cathepsin gene expression were determined in SAE cells following treatment with 1,000 units of recombinant human IFN-β for 24 h. (**d**) Supernatant levels of MMP-8, -9, -10, and -13 following IFN-β stimulation. (**e**) MMP and cathepsin gene expression was performed on SAE cells transfected with silencing RNA for IFN-α receptor 1 (IFNAR1), IFNAR2 or both and treated with RSV. Immunoblots were performed to determine protein silencing compared with actin and two individual transfections are presented. All graphs are represented as mean ± s.e.m., where $n = 10$ per group. *Represents a P-value < 0.05 compared with empty vector-transfected cells.

RSV-induced TLR3 is regulated by RIG-I-dependent IFN-β that is mediated by both IFN response-stimulated element and signal transducer and activator of transcription sites in its proximal promoter.[31] In this manner, TLR3 does not affect RSV-induced NF-κB binding,[31] which may alter the regulation of certain proteases. RIG-I-regulated IFN-β secretions from infected cells could lead to the Trif activation observed here and in the induction of IFNAR1 and IFNAR2 receptors, which



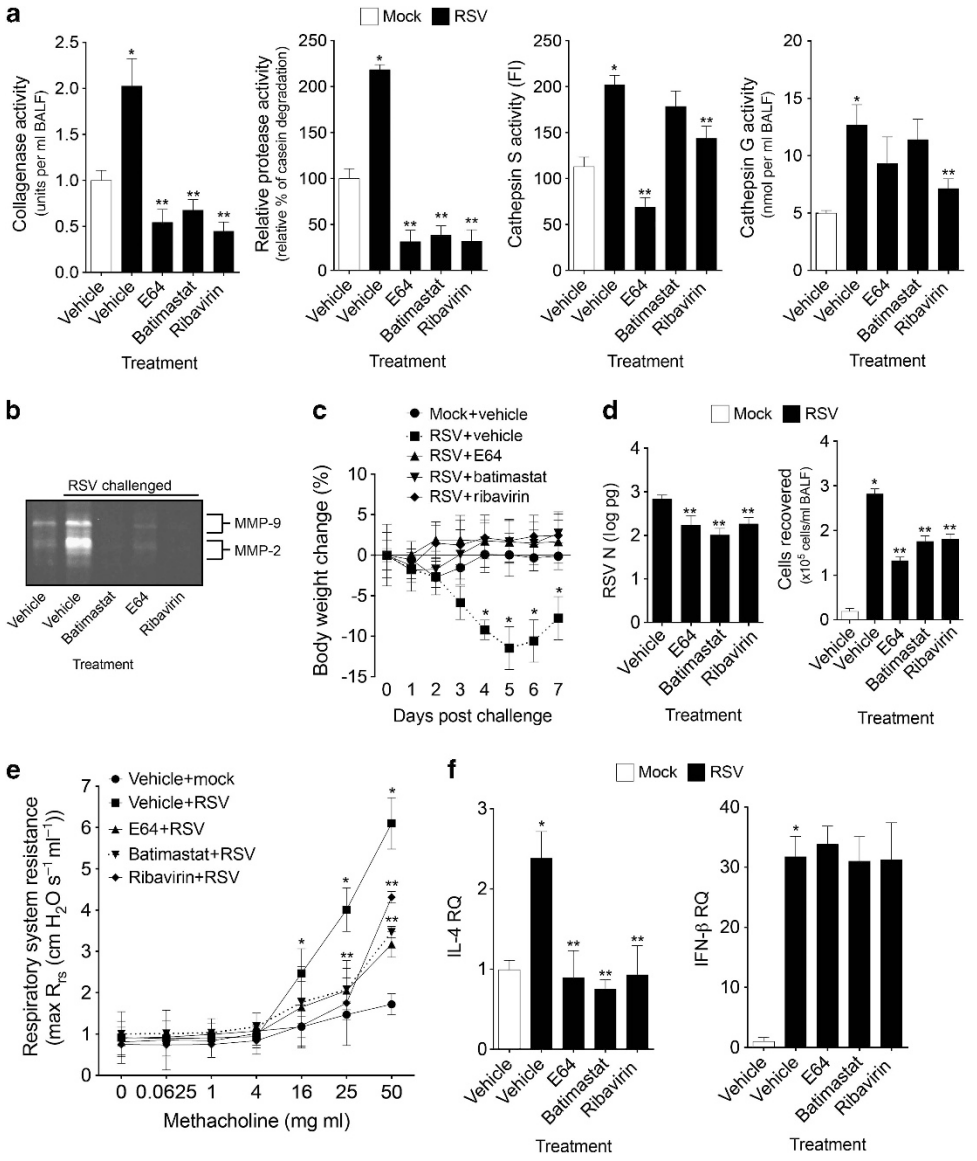


Figure 9 Administration of protease inhibitors or ribavirin aid in respiratory syncytial virus (RSV) clearance and subdue airway hyperresponsiveness in mice. Friend leukemia virus B sensitive strain mice were injected with vehicle, bastimastat, E64, or ribavirin prior to infection with 1×10^6 plaque forming units of RSV and animals received daily follow-up injections until they were killed at day 7 post infection. Bronchoalveolar lavage fluid (BALF) (**a**) collagenase, casein degradation capability, cathepsin S, and G—elastase activity and (**b**) gelatinase activity were determined in all animals groups. (**c**) Animal body weight, (**d**) BALF cellularity and RSV N copy number were determined in all animals. (**e**) Airway hyperresponsiveness to increasing doses of methacholine was assessed in each animal group. (**f**) Interleukin (IL)-4 and intereferon beta (IFN-β) gene expression in lung tissue was analyzed by quantitative PCR. Graphs are represented as mean ± s.e.m., where each measurement was performed three times on 12 animals per group. * and ** represents *P*-values < 0.05 compared with mock and vehicle-treated mice or RSV- and vehicle-treated mice, respectively.

represent two plausible and distinct mechanisms to induce protease production. Therefore, MAVS not only links RIG-I and MDA5 to antiviral effector responses, MAVS can also regulate the RIG-I-inducible proteases response to RSV observed in this study.

Recently, investigators have identified several proteases associated with RSV infection, such as MMP-2, -3, -9, and -10.[8–11] We have identified that RSV enhances MMP-2, -3, -7, -8, -9, -10, -12, -13, -14, -16, -17, -19, -20, -25, -27, -28 and cathepsins B, C, E, G, H, K, L1, S, W, and Z levels in mouse airways. Whether all of these proteases are present in human viral exacerbations or contribute to disease progression is not yet known but each protease represents many potential future topics of interest. Also it should be noted that RSV gave a robust MMP-1 induction in SAE cells, which could not be evaluated *in vivo,* as mice express no MMP-1. Indeed, the protease profile in mice and epithelial cells differed with expression for MMP-2, -14, -17, -20, -25, -27, -28 and cathepsins B, C, H, L1, S, W, and Z, but it is not surprising as other residential cells or infiltrated immune cells could also contribute to protease production in response to RSV. The functional role of proteases in airway remodeling is well

documented[5] but the impact of so many proteases on the microenvironment of the lung is unclear. Our data demonstrates that inhibition of protease activity aids in viral clearance and others have demonstrated that inhibition of MMP-9 activity prevents syncytia formation and blocks RSV multiplication,[10] which suggests that resolving the select protease activity in RSV infection is beneficial. These data strengthen the case for the use of protease inhibitors to treat RSV infections. Identifying the exact protease(s) responsible for aiding viral infectivity represents a major future area of investigation. Ribavirin administration to mice prevented the activity of proteases and also gene expression of several proteases, including MMP-9, -10, -12 and cathepsins B, C, and S (**Supplementary Figure S4**). Expression of MMP-9 represents the most likely candidate for the changes in the phenotype observed in our study as MMP-9 KO mice have reduced airway hyperresponsiveness,[32] and inhibition of MMP-9 blocks RSV[10] and fellow Paramyxoviruses such as the Sendai virus.[33]

Other viruses have been documented to induce protease responses. Indeed, the induction of MMP-9 by Influenza A virus was associated with severe lung pathology in mice[34] and the regulation of neutrophil migration.[35] Also, MMP-9 is higher in children with mumps meningitis.[36] Sendai virus increases MMP-9 activity to drive pneumonia progression by enhancing virus multiplication and aids in the destruction of lung matrix in rats.[33] Whether other viruses lead to the induction of similar host proteases as the RSV or play a similar role in disease progression is a major topic for discussion. Our *in vitro* RIG-I overexpression experiment suggests that multiple viruses, which trigger a RIG-I response, may observe a similar protease response. Also, polyinosinic–polycytidylic acid triggers the expression of multiple MMPs,[15] which further suggests the potential for similar mechanistic protease induction. Other pathogen recognition receptor ligands can induce expression of proteases, such as lipopolysaccharide induction of MMP-1[14,15] or the induction of multiple TLR ligands involved in MMP-9 expression.[14] Therefore, the protease profile induced by other microorganisms and the role of subsequent induced proteases in disease progression remains to be an intriguing area for future study. RSV could also induce many other proteases outside of the MMP and cathepsin families. Neutrophil elastase has been documented to be elevated in the upper airways of infants infected with RSV.[26] Similarly, we observed increased elastase activity following RSV infection, which may not only be due to the increased MMP-12 levels. Interestingly, RSV does induce ADAM17 in mouse airways (**Supplementary Figure S5A**). RSV induction of membrane localized ADAM protease activity has been linked to the activation of extracellular signal-regulated kinase and IL-8 production in a EGFR-dependent manner.[37] In our *in vitro* models RSV also induced emmprin, an inducer of MMP synthesis (**Supplementary Figure S5B**). Emmprin expression affects the production of MMPs[38] and its expression has been linked to pulmonary fibrosis progression[39] and may contribute to the signaling outlined in this study. Therefore it is conceivable that other families of proteases may also become induced in viral infections and lead to the induction of multiple signaling pathways, which would represent an area that merits further investigation.

Neutrophil-derived proteases have an important role in micro-flora clearance, with mice deficient in neutrophil granule proteases unable to resist staphylococcal and candidal infections.[3] However little is known about the antimicrobial functions of protease networks, especially those associated with viral infection. Prior to this study it was unclear whether the host protease response was required to clear the microbial infection or whether the protease release would have an overall negative impact on lung inflammation and remodeling. Due to the numerous proteases induced by RSV, it is out of the scope of this manuscript to investigate the functional role of each protease in response to RSV, but through the use of broad-spectrum protease inhibitors we have determined the impact of proteases on RSV infection and airway hyperresponsiveness to metacholine challenge. Further work is required to determine whether the inhibition of IL-4 by protease inhibitors is the primary impact on preventing RSV-induced airway hyperresponsiveness by protease inhibitors or ribavirin. As IL-4 has a major role in allergic response to RSV infection through the development of airway hyperresponsiveness and lung eosinophilia;[25] it is conceivable that protease regulation of IL-4 may have a major role in disease symptoms. The beneficial effects of inhibiting cathepsins was surprising as cathepsin activity regulates TLR9 interaction with CpG, thereby regulating TLR9 immunity.[40] However recent evidence suggests that cathepsin S can lead to the formation of soluble TLR9 that inhibits TLR9 signaling.[41] Cathepsin S activity was inhibited by E64 and ribavirin. In fact, ribavirin inhibited cathepsin S gene expression. Recent studies suggest that several of the proteases highlighted in this study could contribute to macrophage migration, invasion, differentiation, and maturation, such as MMP-10,[42] MMP-19,[43] and MMP-28.[44] However, further studies are required to investigate their functional roles here.

In summary, our studies demonstrate that RSV acts in a RLR-dependent manner, most likely via RIG-I signaling via type-I IFN, to augment airway protease responses in the lung. Inhibition of protease activity prevented RSV-induced airway hyperresponsiveness and lead to enhanced clearance of the virus. These findings provide important new insights into the elaborate network of proteases triggered by RSV lung infection.

METHODS

RSV culture. Human RSV strain A2 (#VR-1540; ATCC, Manassas, VA) was cultured and virus titers were quantified in Hep2 cells by performing plaque assays, as previously described.[1] Briefly, the virus was allowed to grow for 5 days at 37 °C in a 5% CO_2 atmosphere. The infected Hep2 monolayers were collected and the virus was released by sonication. Cell debris was removed by centrifugation at 2,500 *g* for 5 min at 4 °C. Virus was collected by centrifuging the supernatant for 2 h at 22,000 *g* at 4 °C. Virus were suspended in culture media and snap frozen and maintained at −80 °C. Noninfected Hep2 cell cultures were processed in the same manner as RSV-infected cells and the resulting sample collection was used as a mock control.

Animal models. *Trif* (*Ticam1*) and *Mavs* KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred on to a Friend leukemia virus B sensitive strain background for at least six generations. All mice were maintained in a specific pathogen-free facility at the Mount Sinai Roosevelt's Hospital. Eight-week-old mice were used at the initiation point for all experiments and each experimental parameter had 12 animals per group. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine. Animals were intranasally administered 1×10^6 plaque forming units of RSV or mock. Mock and RSV infection animals were killed every second day up to 9 days post challenge. Animals were weighed daily. BALF and lung tissue were collected for analysis of protease levels. Immune cell counts were determined by microscopy and cell type determined by quick diff staining. All animal experiments were performed with the approval from the Mount Sinai Roosevelt's Hospital Center's Institutional Animal Care and Use Committee approval at the Mount Sinai School of Medicine.

Lung titers and RSV N copy number. Lungs of infected mice were excised and homogenized using a mechanical homogenizer (Kinematica, Bohemia, NY). The viral titers in the homogenates were quantified by plaque assay on Hep2 cells, as previously described.[1] The concentration of RSV N (pg) was determined by PCR based on a standard curve. The following primers were used at 100 pmol each: 5′-TGG GAG AGG TAG CTC CAG AA-3′ and 5′-AGA ATC TGT CCC CTG CTG CTA-3′. Cycle threshold was plotted against known RSV standards. Results are represented as natural log pico grams.

Cell culture. Monolayers of human SAE cells from healthy subjects (Lonza, Walkersville, MD) were submerged cultured. All cells are assayed and tested negative for HIV-1, mycoplasma, Hepatitis-B, Hepatitis-C, bacteria, yeast, and fungi upon isolation by Lonza. Cells were only used for experiments at passages 3–6 and at a confluency of ~70%. SAE cells were transfected by administering siRNA for MDA5, RIG-I, Trif, MAVS, LGP2, IFNAR1, IFNAR2, or control siRNA (Qiagen, Gaithersburg, MD). Cells were treated with RSV at a multiplicity of infection of 0.3 for 24 h. Cells were also treated with mock control as described above. In addition, SAE cells were transfected with either an empty vector (pCMV6-Entry; Origene, Rockville, MD) or with the same vector that overexpresses *RIG-I* (*DDX58*) as recommended by the manufacturers (Origene). Human recombinant IFN-β protein (EMD Millipore, Billerica, MA) was administered to SAE cells at 1,000 U for 24 h.

MMP and cathepsin measurements. MMP and cathepsin gene expression was performed by qPCR using validated Taqman probes (Life Technologies/Applied Biosystems, Carlsbad, CA). All qPCR results are represented as relative quantification compared with the mock-treated animals and corrected to actin levels. Several MMP levels were measured in BALF and cell culture supernatants using a bead assay (MILLIPLEX MAP MMP Magnetic Bead Panels; EMD Millipore) with the Bio-Rad Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA). Cathepsin tissue levels were determined by immunoblots and BALF cathepsin S and B activity assays as previously described.[45] Cathepsin G BALF activity was determined by a commercial available colorimetric assay (ab126780; Abcam, Cambridge, MA). BALF collagenase activity was determined by a colorimetric ninhydrin method, as previously described.[22] Proteinase activity was also assessed with the same colorimetric ninhydrin method but utilizing casein as the substrate. BALF elastase activity was measured by the hydrolysis of N-succinyl-L-Ala-L-Ala-L-Ala-p-nitroanilide per minute at 25 °C and pH 8.0. BALF gelatinase activity was determined by gelatin zymography.

Intracellular signaling. Lung tissue protein from mice was homogenized in radio-immunoprecipitation assay buffer, centrifuged at 13,000 *g* for 10 min and the supernatants were collected. Immunoblots were conducted to determine levels of cathepsins E, S, G, K, B, W, Z (all cathepsin antibodies where purchased from Santa Cruz Biotechnology, Paso Robles, CA), MDA5, RIG-I, MAVS, Trif, MyD88, phosphoryated-IFN regulatory factor 3(Ser396), IFN regulatory factor 3, phospho-TANK-binding kinase 1(Ser172), TANK-binding kinase 1/NAK, and actin (all remaining antibodies where purchased from Cell Signaling Technologies, Danvers, MA). Chemiluminescence detection was performed using the Molecular Imager ChemiDoc XRS+ imaging system (Bio-Rad Laboratories). Densitometry was performed on each target and represented as a ratio of pixel intensity compared with actin, using Image Lab software (version 4.0, build 16; Bio-Rad Laboratories). NF-κBp65 and activator protein 1 levels were measured in the nuclear protein extracts using specific activation assays from Active Motif (46096 and 40096; Active Motif, Carlsbad, CA). Relative transcription factor activity is represented as percentage compared with the mock-treated group.

Immunofluorescence. Immunoreactivity assays were performed on SAE cells with polyclonal anti-RSV (ab20745; Abcam), anti-MMP-9 (#2270; Cell Signaling Technologies), and anti-cathepsin E (sc-166343; Santa Cruz Biotechnology) antibodies. Mouse lung tissue was also examined for RSV immunoreactivity.

Airway responses to methacholine challenge. Airway responses to methacholine (Sigma Chemical, St. Louis, MO) were assessed with the Scireq Flexivent system (Scireq, Montreal, QC, Canada) at 1-week post RSV challenge, and daily intraperitoneal injections of vehicle (20% dimethylsulphoxide), 60 mg kg^{-1} batimastat, 5 mg kg^{-1} E64 or 30 mg kg^{-1} ribavirin. Animals were anesthetized with ketamine/xylazine (10 mg kg^{-1}) and paralysis was induced with 1 mg kg^{-1} pancuronium bromide intraperitoneal (Sigma). The linear single-compartment model was used to assess total respiratory system resistance. Methacholine dose responses were determined.

Statistical analyses. For statistical analysis, data from 12 animals or multiple separate cell experiments were pooled. Data are expressed as means ± s.e.m. Differences between groups of mice over time were compared by two-way analysis of variance. Individual differences between groups were tested by multiple comparison and analysis using the Bonferroni post-test. Pairs of groups were compared by Student's *t*-test (two tailed). *P*-values for significance were set at 0.05. All analysis was performed using GraphPad Prism Software (Version 5 for Mac OS X, GraphPad Software, Inc., La Jolla, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declare no conflict of interest.

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