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The ASK family kinases differentially mediate type Ⅰ interferon induction and apoptosis during the antiviral response

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Abstract

Viral infection activates host defense mechanisms, including the production of type I interferon (IFN) and apoptosis of the infected cells. We investigated whether these two antiviral responses were differentially regulated in infected cells. We showed that the mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) Apoptosis signal–

regulating kinase 1 (ASK1) was activated in response to the synthetic double-stranded RNA analog poly(I:C) (polyinosinic-polycytidylic acid) and RNA virus, and that it played an essential role in inducing both IFNB gene and apoptotic cell death. We further showed that the MAPKKK ASK2, a modulator of ASK1 signaling, which forms hetero-oligomers with ASK1, was essential for ASK1-dependent apoptosis, but not IFNB gene induction. Furthermore, genetic deletion of either ASK1 or ASK2 in mice promoted the replication of influenza A virus in the lung. Our study therefore identifies ASK1 and ASK2 as components of the antiviral defense mechanism, and suggests that ASK2 acts as a key modulator that promotes apoptosis rather than the type I IFN response. Because ASK2 is selectively expressed in epithelium-rich tissues such as the lung, ASK2-dependent apoptosis may contribute to an antiviral defense in tissues with a rapid repair rate where apoptotic cells are replaceable.

Introduction

Mammalian cells deploy the innate immune system as the first line of defense against viruses. The type I interferons (IFNs) IFN-α and IFN-β play a central role in this innate immune response by activating the expression of hundreds of IFN-stimulated genes (ISGs) whose products establish an "antiviral state" to restrict viral replication within infected cells. Type I IFNs also promote the proliferation of effector lymphocytes to provide long-term and specific protection against the infecting virus through the production of various cytokines and chemokines, such as interleukin-15 (IL-15) (*1*). Thus, regulation of the expression of genes encoding type I IFNs is the subject of intense investigation. Cytoplasmic double-stranded RNA (dsRNA), a common byproduct of the replication of DNA and RNA viruses, is recognized by the retinoic acid–inducible gene I (RIG-I)–like helicase receptors (RLRs) RIG-I and melanoma differentiation–associated gene 5 (MDA5) (*2, 3*), which mediate the expression of genes encoding type I IFNs through their interaction with their common adaptor protein interferon-β promoter stimulator 1 (IPS-1) (also known as MAVS, VISA, or CARDIF) (*4-7*) and the subsequent tumor necrosis factor (TNF) receptor–associated factor

(TRAF)-mediated activation of its downstream effectors, the transcription factors interferon regulatory factor–3 (IRF-3) and nuclear factor κB (NF-κB) (*8, 9*). In addition to having IRF3- and NF-κB–binding sites, the promoter of the gene encoding *IFNB* contains an activator protein 1 (AP-1)–binding site that is essential for gene expression. Indeed, the AP-1 complex, which consists of c-Jun and activating transcription factor 2 (ATF-2), as well as the upstream activating mitogen-activating protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK) are necessary for the effective induction of *IFNB* in response to cytoplasmic dsRNA (*10-13*). However, although various candidates have been proposed, the molecules that mediate activation of p38 and JNK in response to cytoplasmic dsRNA are unclear.

In addition to the induction of type I IFN production, another main viral defense strategy of the innate immune response is the induction of apoptosis (*14, 15*). Given that viruses need host cells within which to replicate, the elimination of infected cells prevents further spread of infection. Many viruses have evolved mechanisms to interfere with host cell apoptosis despite their being subjected to rigorous evolutionary selection to maintain small genomes, which reflects the importance of this strategy in antiviral immunity. Indeed, prevention of apoptosis, for example by deletion of the gene encoding Bax, a prapoptotic B-cell lymphoma 2 (Bcl-2) family member that triggers mitochondrial apoptosis, results in enhanced viral replication and pathogenesis in mice (*16*). Characterization of the mechanism underlying virus-induced apoptosis is thus critical to understanding antiviral host defense. As well as enabling type I IFN production, RLRs and IPS-1 mediate the induction of caspase activation and apoptosis in response to cytoplasmic dsRNA (*17-19*). However, although IFN-β promotes apoptosis through p53-dependent signaling (*20*), both IFN-β and p53 are, at least in some cases, not required for apoptosis induced by cytoplasmic dsRNA and IPS-1 (*21, 22*). IRF3 is implicated in the induction of apoptosis by IPS-1 in some instances (*16, 23*), but not in others (*18, 19*). The effectors of IPS-1–induced apoptosis thus remain to be elucidated. Although apoptosis appears to be an effective means of suppressing viral replication, the elimination of cells can be damaging to the organism. The type I IFN response can also be harmful in some instances (*24-28*). Therefore, it is reasonable to assume that host cells may differentially regulate the production of type I IFN and the induction of apoptosis to optimize

the benefit to the organism in a context-dependent manner. It remains unclear whether such differential regulation exists and, if it does, how it is controlled.

The activities of the MAPKs JNK and p38 are strictly regulated by their upstream MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs) (*29*). Among a number of MAPKKKs, Apoptosis signal–regulating kinase 1 (ASK1) is an evolutionarily conserved enzyme that activates both JNK and p38 through the MAPKKs MEK3, MEK4, MEK6, or MEK7 (*30*). ASK1 mediates the induction of apoptosis in response to various stimuli, including oxidative stress, endoplasmic reticulum (ER) stress, and TNF-α (*31*). ASK1 is part of a large complex (>1500 kD) known as the ASK1 signalosome, which includes TRAF2 and TRAF6 (*32*). ASK2, a MAPKKK closely related to ASK1, forms a hetero-oligomer with ASK1 and promotes its pro-apoptotic function (*33, 34*). Studies have revealed that mouse ASK1 and its *Caenorhabditis elegans* ortholog Neuronal Symmetry-1 (NSY-1) both mediate the innate immune response to bacteria (*35, 36*). Here, we showed that ASK1 mediated the activation of p38 and JNK in response to synthetic double-stranded RNA analog poly(I:C) (polyinosinic-polycytidylic acid) and RNA viruses, and acted as a link between IPS-1 activation and the AP-1–dependent expression of the gene encoding IFN-β. We found that ASK1 stimulated both IFN-β gene induction and apoptosis in response to poly(I:C) and RNA viruses, and thereby played an essential role in preventing viral spread. Furthermore, our results indicate that ASK2 differentially regulated type I IFN production and apoptotic responses and suggest that ASK2 may thereby determine the cellular outcome of viral infection.

Results

RLRs, IPS-1, and TRAF family proteins connect the pathway involved in sensing of cytoplasmic dsRNA with the p38 and JNK pathways

To dissect the signaling pathway upstream of p38 and JNK in the viral induction of the *IFNB*, we first asked whether RLRs and their adaptor IPS-1 were involved. Through Western blotting analysis (Fig. 1, A to C and fig. S1), we found that knockdown of RIG-I or MDA5 by expression of the corresponding short-hairpin RNA (shRNA) inhibited the activation of the p38 and JNK pathways in HeLa S3 cells in response to their cognate stimuli, that is, poly(I:C), a synthetic analog of dsRNA, and encephalomyocarditis virus (EMCV) for MDA5, and Newcastle disease virus (NDV) for RIG-I (*37*). Knockdown of IPS-1 also inhibited the activation of the p38 and JNK pathways in response to transfection with poly(I:C) or infection with EMCV or NDV (Fig. 1, B and C, and fig. S1), whereas overexpression of wild-type IPS-1 in 293T cells (Fig. 2, A and B) increased the activity of a reporter gene construct containing the promoter domain IV of the *IFNB* gene (PRD4-Luc), which is activated by ATF-2 and c-Jun (Fig. 2B). These results suggest that RLRs and IPS-1 are necessary for the effective activation of the p38 and JNK pathways in response to viral infection and dsRNA.

TRAF family proteins are capable of the activation of IRF3 and NF-κB downstream of IPS-1 (*7-9*). Because TRAF2, TRAF3, TRAF5, and TRAF6 interact with IPS-1 and are capable of activating the p38 and JNK pathways (*38*), we examined the possible involvement of the TRAF-binding sites of IPS-1 (Fig. 2A). We found that whereas overexpression of IPS-1 containing a single mutation in any one of the TRAF-binding sites increased the activity of the PRD4-Luc reporter construct similarly to that in cells expressing comparable amounts of wild-type IPS-1, overexpression of IPS-1 containing mutations in all three TRAF-binding sites (the IPS-1 dT2/dT3/dT6 mutant) (*7, 8*) failed to increase the activity of PRD4-Luc and also suppressed the induction of *IFNB* expression in cells transfected with poly(I:C) (Fig. 2, B and C). Furthermore, knockdown of either TRAF2 or TRAF6 partially suppressed the

phosphorylation of p38 and JNK in cells transfected with poly(I:C), and simultaneous knockdown of both TRAF2 and TRAF6 further suppressed the phosphorylation of p38 and JNK, as well as of ATF-2 and c-Jun (Fig. 2D and fig. S2, fig. S3). On the other hand, knockdown of TRAF3 had almost no effect on the extent of phosphorylation of p38 and JNK in response to transfection with poly(I:C) (fig. S4). These data suggest that TRAF2 and TRAF6 are involved in the IPS-1–dependent activation of p38 and JNK signaling.

ASK1 mediates activation of p38 and JNK signaling and *IFNB* gene in response to poly(I:C) and viral infection

We next examined potential mediators of the viral infection–induced activation of the p38 and JNK pathways that functioned downstream of TRAF2 and TRAF6. Among the large number of MAPKKKs that activate p38 and JNK, we focused on ASK1, which functions as a downstream effector of TRAF2 and TRAF6 in other contexts (*32, 39*). To determine whether ASK1 was activated in HeLa S3 cells in response to transfection with poly(I:C), we performed Western blotting analysis with an antibody specific for phosphorylated (and thus activated) ASK1 (*40*). We found that poly(I:C) dose-dependently stimulated the phosphorylation of ASK1, p38, and JNK, indicating that ASK1 was activated by cytoplasmic dsRNA (Fig. 3A). Consistent with these results, both infection with EMCV and overexpression of IPS-1 also resulted in an increase in ASK1 phosphorylation (Fig. 3, B and C). Furthermore, we found that endogenous IPS-1 co-immunoprecipitated with ASK1 in HeLa S3 cells in response to poly(I:C) (Fig. 3D). Together with previous data (*32, 39*), these results suggest that ASK1 is commonly activated in response to viral infection and poly(I:C) through its interaction with IPS-1 and TRAFs.

We next investigated whether ASK1 was required for the induction of *IFNB* expression by

poly(I:C). Knockdown of ASK1 substantially suppressed the poly(I:C)-stimulated phosphorylation of ATF-2 and c-Jun and the expression of *IFNB*, but not the dimerization of IRF3 or the degradation of inhibitor of κB α (IκBα) (Fig. 4, A to C), which reflect activation of IRF3 and NF-κB, respectively. These results suggest that ASK1 stimulates *IFNB* expression by activating the p38 and JNK pathways, but not the IRF3 or NF-κB pathways. We also found that knockdown of ASK1 impaired both the increase in *IFNB* mRNA abundance and the activation of MAPK pathways in response to infection with EMCV, NDV, or influenza A virus CA04 (Fig. 4, A and C, and fig. S3, fig. S5), further supporting the notion that ASK1 is necessary for the induction of *IFNB* expression, acts upstream of p38 and JNK, and acts downstream of RLRs. Experiments with mouse embryonic fibroblasts (MEFs) from $ASK1^{+/+}$ and ASK1-deficient ($ASK1^{-/-}$) mice produced similar findings (Fig. 4, D and E). These results suggest that ASK1 is indeed necessary for the efficient induction of *IFNB* expression by poly(I:C).

To understand the importance of ASK1 in suppressing viral replication, we quantified viral RNA abundance after infection of MEFs from wild-type and $ASK1^{-/-}$ mice with NDV. We found that NDV RNA was substantially greater in abundance in the $ASK1^{-/-}$ MEFs than in the wild-type MEFs (Fig. 4F). We also used a modified Sendai virus (SeV) encoding green fluorescent protein (SeV-GFP), which enabled us to directly visualize viral replication, and we found that $ASK1^{-/-}$ MEFs exhibited enhanced SeV-GFP replication compared to that in wild-type cells (Fig. 4G). These data suggest that ASK1 is a previously uncharacterized component of the RLR pathway and that it plays a role in suppressing viral replication.

ASK1 is necessary for apoptosis induced by cytoplasmic dsRNA

Relatively little is known about the mechanism that regulates apoptosis in response to

cytoplasmic dsRNA or viral infection, although IRF3 and p53 are thought to be involved (*16, 20, 23*). Because ASK1 executes apoptosis induced by various types of stress (*31*), and because it is also implicated in the mediation of influenza virus–induced apoptosis through ER stress (*41*), we next asked whether ASK1 mediated apoptosis in response to poly(I:C). Transfection of control cells with poly(I:C) induced chromatin condensation, phosphatidylserine exposure, and cleavage of caspase-3, which are all indicators of apoptosis, whereas knockdown of ASK1 reduced the extent of these responses (Fig. 5, A to C). These results suggest that ASK1 is necessary for effective induction of apoptosis by cytoplasmic dsRNA. Because this role of ASK1 was observed in HeLa S3 cells in which p53 activity was suppressed by papillomavirus E6 protein, ASK1 appeared to mediate dsRNA-induced apoptosis in a p53-independent manner. We next examined whether ASK1 also mediated caspase activation in response to viral infection. Infection of HeLa S3 cells with NDV, EMCV, or infection of A549 cells with influenza virus increased the extent of cleavage of caspase-3 (Fig. 5C and fig. S5), whereas knockdown of ASK1 impaired the cleavage of caspase-3 induced by any of these viruses, suggesting that ASK1 is necessary for the effective induction of apoptosis in response to viral infection. Together with the finding that ASK1 was dispensable for the poly(I:C)-induced activation of IRF3, these results suggest that ASK1 mediates apoptosis independently of IRF3 activation at least in this system.

ASK1 mediates antiviral responses in vivo

To assess the involvement of ASK1 in antiviral responses in vivo, we infected ASK1$^{+/+}$ and ASK1$^{-/-}$ mice with the influenza A virus (Fig. 6, A to C and fig. S6). The abundance of *IFNB* mRNA was substantially increased in the lungs of ASK1$^{+/+}$ mice, but not ASK1$^{-/-}$ mice, after infection with influenza A virus(Fig. 6A). Next, we examined whether ASK1 was involved in the induction of apoptosis in response to viral infection in vivo. Through

immunohistochemistry, we labeled virus-infected epithelial cells in the bronchioles of ASK1$^{+/+}$ mice with an antibody specific for the viral antigen M1. We observed that a considerable number of these infected cells displayed activation of caspase-3 after viral infection, although caspase-3 activation in uninfected cells was almost undetectable (Fig. 6B). These results suggest that bronchiolar epithelial cells undergo apoptosis as an early response to influenza virus. In contrast, the percentage of M1-positive bronchiolar epithelial cells that were also positive for active caspase-3 in ASK1$^{-/-}$ mice was substantially lower than that in ASK1$^{+/+}$ mice (Fig. 6B), suggesting that ASK1 was necessary for the effective activation of caspases in vivo. Consistent with these results, the viral titer in influenza virus–infected lungs was markedly greater in ASK1$^{-/-}$ mice than in ASK1$^{+/+}$ mice (Fig. 6C). These results suggest that ASK1 mediates antiviral responses in vivo. We also found that the loss of ASK1 substantially increased morbidity after the infection of mice by EMCV (Fig. 6D), and that the viral titer was greater in ASK1$^{-/-}$ mice than in ASK1$^{+/+}$ mice (Fig. 6E). This finding further emphasizes the role of ASK1 in mediating antiviral host defense.

ASK2 is necessary for apoptosis, but not *IFNB* expression, in response to poly(I:C)

That ASK1 mediated the induction of both *IFNB* expression and apoptosis in response to viral infection or poly(I:C) led to the question of how cells discriminate between these two ASK1-mediated responses. To investigate this question, we examined possible roles for ASK2, a modulator of ASK1 signaling, which forms hetero-oligomers with ASK1 (*33*). Knockdown of ASK2 in HeLa S3 cells or A549 cells did not reduce the extent of induction of *IFNB* expression in response to poly(I:C), EMCV infection, or influenza A virus infection (Fig. 7, A and B, fig. S7, and fig. S8). In contrast, knockdown of ASK2 substantially suppressed the extent of poly(I:C)-induced chromatin condensation and phosphatidylserine exposure (Fig. 7, C and D). Furthermore, knockdown of ASK2 impaired the cleavage of

caspase-3 in HeLa S3 cells or A549 cells in response to poly(I:C) and viral infection (Fig. 7E, fig. S7, and fig. S8). These results suggest that hetero-oligomers of ASK1 and ASK2 are necessary for the effective induction of apoptosis by poly(I:C) and viral infection, but not for the expression of *IFNB*.

A high abundance of ASK2 confers the potential to undergo apoptosis in response to viral infection and poly(I:C)

Because ASK2 was necessary for the induction of apoptosis by poly(I:C), but was dispensable for the induction of *IFNB* expression, we hypothesized that ASK2 determined which of these two ASK1-mediated host defense strategies was adopted. To investigate whether the amount of ASK2 was a critical determinant of apoptosis, we overexpressed ASK2 with a tetracycline-inducible (Tet-ON) system in a stable human embryonic kidney (HEK) 293 cell line expressing a kinase-defective form of ASK1, because the stability of ASK2 depends on a kinase-independent function of ASK1, and ectopic expression of ASK2 alone is not sufficient (*33*). An increase in the abundance of ASK2 enhanced the activation of caspase-3 in response to poly(I:C), NDV infection, or EMCV infection (Fig. 7F and fig. S9), but it did not enhance the induction of *IFNB* expression under the same conditions (Fig. 7G and fig. S9). Together with our observations from ASK2 knockdown experiments, these results suggest that ASK2 is a pivotal regulator of ASK1 whose abundance determines whether an apoptotic response is induced by viral infection.

ASK2 selectively activates an apoptotic response and inhibits viral replication in vivo

Next, we examined the involvement of ASK2 in antiviral responses in vivo. We infected epithelial cells in the lung with influenza A virus, because ASK2 is highly abundant in epithelium-enriched tissues, such as the skin and the lung (*34*), and because this virus mainly

replicates and causes pathological lesions in the lung (*42*). We first examined the extent of expression of *IFNB* in the infected lung. The abundance of *IFNB* mRNA in the lungs of ASK2$^{-/-}$ mice after infection with influenza A virus was comparable to that of infected ASK2$^{+/+}$ mice (Fig. 7H), suggesting that ASK2 was dispensable for activation of *IFNB* expression in response to viral infection in vivo. However, consistent with the in vitro experiments, the percentage of bronchiolar epithelial cells positive for active caspase-3 among those positive for the viral protein M1 in ASK2$^{-/-}$ mice was substantially reduced compared to that in infected ASK2$^{+/+}$ mice (Fig. 7I). This result suggests that ASK2 was necessary for the effective activation of caspase-3 in vivo. Finally, we examined a potential role for ASK2 in viral spreading. We found that the viral titer in the lungs of ASK2$^{-/-}$ mice was substantially greater than that in the lungs of infected ASK2$^{+/+}$ mice (Fig. 7J), although the amounts of *IFNB* mRNA were comparable. Together, these results suggest that ASK2 is a pivotal antiviral protein that selectively contributes to the activation of an apoptotic response in vivo and inhibits viral propagation in the lung, an epithelium-rich tissue with a rapid repair rate.

Discussion

Here, we identified ASK1 as a previously uncharacterized mediator of the host cell antiviral response. Our results suggest that ASK1 has two important functions in the restriction of viral infection: induction of *IFNB* expression and induction of apoptosis. This functional bifurcation (one molecule, two functions) is expected to be beneficial if both functions are concertedly or differentially regulated through this molecule. Indeed, our data suggest that the ASK1-binding partner ASK2 contributes to the choice between these functions of ASK1 by promoting ASK1-mediated apoptosis. Our study thus unveils the existence of cellular machinery that discriminates between two host defense strategies, which may serve as a point

of their differential regulation (fig. S10).

Our results suggest that ASK2 plays an essential role in inducing apoptosis, but not *IFNB* expression, in response to cytoplasmic dsRNA. Because ASK2 does not form homo-oligomers, but instead forms hetero-oligomers with ASK1, it appears likely that ASK1 homo-oligomers promote the production of type I IFN, whereas ASK1-ASK2 hetero-oligomers promote apoptosis. ASK2 thus serves as a molecular switch that modulates the functions of ASK1 in antiviral responses. The mechanism by which ASK1 homo-oligomers and ASK1-ASK2 hetero-oligomers induce different outcomes despite their close structural similarity remains unknown. However, we and others have observed that increases in the abundance of ASK2 result in more pronounced and sustained activation of JNK than of p38, and that sustained activation of JNK is associated with apoptosis (*33, 43*), implying a role for JNK-induced apoptosis in ASK2-specific functions. To examine the interaction between ASK1 and ASK2, we also performed coimmunoprecipitation experiments and found that the amount of coprecipitated ASK2 with ASK1 did not markedly change after poly(I:C) transfection (fig. S11). This is consistent with a previous study showing that ASK1 and ASK2 form a stable and inactive complex at a basal level and that

the activity of this complex is regulated by some other factors (*32*). The mechanism by which ASK1/ASK2 hetero-oligomer activated upon viral infection is still an open question.

In which contexts would these antiviral strategies be differentially used? Although apoptosis eliminates an infected cell, such elimination may be costly for cells that are in limited supply. For example, lost neurons are not normally replaced by new neurons in most parts of the adult brain, so neurons should not easily undergo apoptosis. The benefits of apoptosis outweigh the risks, however, for cells in plentiful supply with a high turnover rate, such as those in epithelial tissues. Indeed, ASK2 is highly abundant in epithelial tissues, but not non-epithelial tissues, whereas ASK1 appears to be ubiquitously expressed (*34*). Consistent with this scenario, deletion of ASK2, which reduced the activation of caspase-3, but not the expression of *IFNB*, enhanced the replication of influenza virus in the lungs, a representative, epithelium-rich tissue with a rapid repair rate (Fig. 7). We therefore speculate that renewable epithelial cells eliminate viruses in part by ASK2-dependent apoptosis, whereas other cell types with less ASK2 abundance eliminate viruses by other means (such as type I IFN production). It is also possible that ASK2-dependent apoptosis takes place in a context in which the production of type I IFN is harmful. Because type I IFN production reduces hematopoietic stem cells and compromises host defense against some bacterial infections (*22, 24-28*), it would be interesting to investigate whether ASK2 plays a role in this context in future studies.

Although the regulation of *IFNB* expression by viral dsRNA has been studied extensively, the signaling pathway that activates p38 and JNK and the AP-1 element of *IFNB* was unclear. Among members of the MAPKKK family, TGF-β activated kinase 1 (TAK1) has been

implicated in the induction of type I IFN production (*44*); however, deletion of the gene encoding TAK1 does not affect type I IFN production in response to cytoplasmic dsRNA or infection with Vesicular stomatitis virus (VSV) (*13, 45*). Knockdown of MEKK1 partially inhibits the production of type I IFN in response to dsRNA, although the activation of MEKK1 by dsRNA has not been described (*13*). Here, we found that ASK1 was phosphorylated at its critical site for activation in response to poly(I:C) or viral infection, and that ASK1 played an essential role in the activation of p38 and JNK signaling and of AP-1, as well as in the expression of *IFNB*. Furthermore, endogenous ASK1 coimmunoprecipitated with IPS-1, a key adaptor protein for RLRs, in response to poly(I:C). A proportion of total cellular ASK1 is thought to be localized at the mitochondria, where IPS-1 resides, consistent with the notion that ASK1 mediates signaling from IPS-1 by forming a protein complex(*46, 47*). Together, these results suggest that ASK1 connects IPS-1 and the p38 and JNK signaling pathways in the viral induction of *IFNB* expression.

A point that still remains to be resolved is how IPS-1 activates ASK1. Our results suggest that TRAF2 and TRAF6, both of which are recruited by IPS-1 to the insoluble fraction that includes the mitochondrial membrane (*7*), play a role in activating ASK1. Given that a proportion of ASK1 and IPS-1 are located at the mitochondrial outer membrane, the association of ligand-bound RLRs with IPS-1 may stimulate the activation of an IPS-1–TRAF–ASK1 complex by inducing conformational changes or through additional modulators. Reactive oxygen species (ROS) increase the activity of ASK1 under oxidative stress by removing the inhibitor thioredoxin from ASK1 (*48*), and IPS-1 induces *IFNB* expression in a ROS-dependent manner (*49*). ROS might therefore contribute to activation of an IPS-1–TRAF–ASK1 complex that is induced by cytoplasmic dsRNA. As a common mediator of cellular stress, ASK1 might sense cellular conditions (such as the extent of oxidative stress)

and modulate the outcome of viral infection.

The history of host-pathogen interactions has given rise to the evolution of viral molecules that antagonize host defense mechanisms. For example, the human immunodeficiency virus (HIV) gene product Nef binds to and inhibits the activity of ASK1 (*50*), which is consistent with the importance of ASK1 in antiviral strategies. Our findings may help to explain how ASK1 restricts the spread of such viruses. Our identification of ASK1 and ASK2 as regulators of the innate immune response and the elucidation of the underlying mechanisms that ASK family kinases differentially mediate type I IFN induction and apoptosis may provide a basis for the development of new therapies for the effective elimination of viruses.

Materials and methods

Plasmids and reagents

The constructs encoding wild-type (WT) human IPS-1 and human ASK1 were described previously (*48, 51*). Site-directed mutagenesis was performed with a QuickChange site-directed mutagenesis kit (Stratagene) to generate mutant human IPS-1 that could not interact with TRAFs, as described previously (*7, 8*). The reporter gene construct containing promoter domain 4 of *IFNB* (PRD4-RLuc) was generated as described previously (*11*). Poly(I:C) was purchased from GE Healthcare. To stimulate cells, poly(I:C) was mixed with Lipofectamine 2000 (Invitrogen) and then added to cells at final concentrations of 0.25, 2.5, or 7.5 μg/ml.

RNA interference

Small interfering (si) RNA vectors were constructed by inserting oligonucleotides into the Bsp MI sites of the pcPUR-U6i expression vector, as described previously (*52*). HeLa S3

cells were transfected with 2.0 μg of siRNA vector in 5.0 μl of Lipofectamine 2000 transfection reagent (Invitrogen). The cells were then used for subsequent assays after incubation for 48 hours in the presence of puromycin (2.0 μg/ml, Sigma). The sequences of the siRNA targeting sequences used in this study are follows: GFP (control), 5'-GGGTGCTCAGGTAGTGGTT-3'; human ASK1-1, 5'-GGAACAGCCTTCAAATCAA-3'; human ASK1-2, 5'-GAAAGAGAAAGAATTACAA-3'; human ASK2-1, 5'-GCCCCGACATCATCATGAA-3'; human ASK2-2, 5'-GACAAAGCGTATTAAACAA-3'; human MDA5-1, 5'-GCGACAAATTTAAATACAT-3'; human MDA5-2, 5'-GCAGAAATGTCCAAATGAT-3'; human RIG-I-1, 5'-GATTGAGAATTTATCACAA-3'; human RIG-I-2, 5'-AGTTAAACATTTAATATGA-3'; human IPS-1-1, 5'-GACAAGACCTATAAGTATA-3'; human IPS-1-2, 5'-GCTGAAGACAAGACCTATA-3'; human TRAF2-1, 5'-GGACCAAGCTGGAAGCCAA-3'; human TRAF2-2, 5'-GCTGAAGACAAGACCTATA-3'; human TRAF3-1, 5'-GTTGCAGAATGAAAGTGTA-3'; human TRAF3-2, 5'-GTGCCAGGGTCTACCTGAA-3'; human TRAF6-1, 5'-GGTGAAATGTCCAAATGAA-3'; human TRAF6-2, 5'-GTTTAAACCCTAAATATAA-3' ; human TLR3-1, 5'-GGAGAAACTTTCTCAATTT-3' ; human TLR3-2, 5'-GTGAAGAACTGGATATCTT-3'. Stealth RNAi (Invitrogen) was used for ASK1 and ASK2 knockdown experiments in A549 cells. A549 cells were transfected with siRNA oligonucleotides with the Lipofectamine RNAiMAX reagent (Invitrogen). The cells were

then used for subsequent assays after incubation for 72 hours. The siRNA sequences were 5'-UGAAGCUAAGUAGUCUUCUUGGUAA-3' and 5'-UUACCAAGAAGACUACUUAGCUUCA-3' for ASK1, and 5'-GAGGUCAGAGGAGCUGAGUAAUGAA-3' and 5'-UUCAUUACUCAGCUCCUCUGACCUC-3' for ASK2, respectively. The negative control Med GC #1 siRNA was used as a control.

Real-time qPCR analysis

Total RNA was obtained from cells with RNAiso (Takara) according to the manufacturer's instructions. Reverse transcription (RT) was performed with 1 μg of total RNA, oligo d(T)12-18 (Invitrogen) primers, and ReverTra Ace (TOYOBO). The resulting complementary DNA (cDNA) was subjected to real-time qPCR analysis in a Roche LightCycler with SYBR Premix Ex Taq (Takara). The abundance of target mRNA was normalized relative to that of *GAPDH* mRNA. The sense and antisense primers, respectively, were as follows: human *GAPDH*, 5'-CTGAACGGGAAGCTCAC-3' and 5'-GTCATCATACTTGGCAGGT-3'; human *IFNB*, 5'-GCTCTCCTGTTGTGCTTCTC-3' and 5'-AGTCTCATTCCAGCCAGTGC-3'; human *ASK2* 5'-TGCTGGTCCTGGAGATGAA-3' and 5'-TCAGGGTCACTGTGCTTAC-3'; human *TLR3* 5'- GAAACTAGAAATTCTCGATTTGCAG-3' and 5'-CAAGTTAAGGATGTGGAGGTGA'; mouse *GAPDH* 5'-ATGAATACGGCTACAGCAACAGG-3' and 5'-CTCTTGCTCAGTGTCCTTGCTG-3'; mouse *IFNB* 5'-TCCACCAGCAGACAGTGTT-3' and 5'-CTTTGCACCCTCCAGTAATAGC-3'; NDV APMV1 5'-AGTGATGTGCTCGGACCTTC-3' and 5'-CCTGAGGAGAGGCATTTGCTA-3'.

Viral infection

SeV-GFP virus was used for infections and was titered as previously described (*53*). Cells were infected with EMCV and NDV as described previously (*3*). Mice were infected with EMCV or Influenza A virus (A/California/04/09; Ca04) and infected lungs were analyzed by standard plaque assays as described previously (*42, 54*). The 50% mouse lethal dose (MLD 50) for Ca04 was $10^{2.8}$ plaque-forming units (p.f.u.).

Mice

The generation of ASK1-deficient (*Map3k5*$^{-/-}$) mice and ASK2-deficient (*Map3k6*$^{-/-}$) mice was described previously (*34, 55*). All mice were maintained according to protocols approved by the Animal Care and Use Committee of the University of Tokyo.

Antibodies

The antibodies used in this study, and their sources, are as follows: anti-ASK1(F9) and anti-ASK1(H300), anti-HA(Y11), anti-IκBα, anti-Myc(9E10), anti-p38, anti-pc-Jun, anti-TRAF2(C-20), and anti-TRAF3(G-6) (Santa Cruz Technology); anti-pp38, anti-pJNK, anti-cleaved-caspase-3, and anti-pIRF-3 (Cell Signaling Technology); anti-Flag(M2) and anti–α-tubulin (Sigma); anti–IPS-1 (Abcam), anti-IRF3 and anti-TRAF6 (MBL), anti-pATF-2 (New England Biolab); and anti-M1 (Serotec). Antibodies specific for pASK1, MDA5, and RIG-I were described previously (*40, 56*).

Cell lines and transfections

HeLa S3 cells, HEK 293T cells, A549 cells, MDCK cells, and MEFs from wild-type mice, ASK1$^{-/-}$ mice, and IFNAR1$^{-/-}$ mice were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). 293-ASK2/1KN cells were maintained

as described previously (*34*). Transfection of HEK 293T cells was performed with the FuGENE6 transfection reagent (Roche).

Western blotting analysis

Western blotting analysis was performed as described previously (*57*). Native polyacrylamide gel electrophoresis (PAGE) to detect IRF3 dimers was performed as described previously (*3*).

Immunohistochemistry

Mouse lungs were inflated with up to 1 ml of 4% paraformaldehyde (PFA) and fixed for 4 hours at 4°C in PFA, cryoprotected in a 10% sucrose solution in phosphate-buffered saline (PBS) at 4°C overnight, followed by overnight incubations at 4°C in 20 and 30% sucrose solutions, and finally embedded and frozen in OCT (TissueTEK). Lungs were then sectioned into 12-μm sections with a cryostat. Sections were exposed to Tris-buffered saline (TBS) containing 0.1% Triton X-100 and 3% bovine serum (blocking buffer), and were first incubated for 24 hours at 4°C with primary antibodies in blocking buffer, and then were incubated for 1 hour at room temperature with Alexa Fluor–conjugated secondary antibodies in blocking buffer. Images were acquired with TCS-SP5 (Leica) confocal microscopes and were processed with Photoshop CS software (Adobe).

Coimmunoprecipitations

We performed coimmunoprecipitations with anti-Flag antibody (M2, Sigma) and anti-HA antibody (Y11, Santa Cruz Technology) for cells ectopically expressing Flag-IPS-1 and HA-ASK1, and with anti-ASK1 antibody (F9, Santa Cruz Technology) and anti-IPS-1 antibody (Abcam) for cells with endogenous ASK1 and IPS-1. The cells were washed with PBS and lysed with the cell-lysis buffer described earlier.

Luciferase reporter analysis

HeLa S3 cells seeded on 48-well plates were transiently transfected with 2 ng of the Renilla luciferase reporter plasmid together with a total of 100 ng of various expression plasmids or empty control plasmids. As an internal control, 20 ng of pGL3-control (Promega) was used. Twenty-four hours later, the luciferase activity was measured with a Dual-Luciferase reporter assay system (Promega).

Annexin-V binding assays

Cells were stained with annexin-V coupled to Cy5 (TAKARA) in accordance with the manufacturer's instructions. Flow cytometric analysis was performed with a BD FACSAria flow cytometer (BD Biosciences).

Statistical analysis

All quantitative data are shown as means ± SD or SEM, unless otherwise noted. Values were compared with unpaired Student's *t* test and log-rank tests. $P < 0.05$ was considered to be statistically significant.

Supplementary Materials

Fig. S1. The RLR pathway is necessary for the activation of IRF3 after infection with EMCV.
Fig. S2. TRAF2 and TRAF6 mediate the activation of ATF-2 and c-Jun in response to poly(I:C).
Fig. S3. The quantification of several western blots presented in the manuscript.
Fig. S4. Analysis of the effect of TRAF3 knockdown on the activation of the p38 and JNK pathways.
Fig. S5. ASK1 is necessary for effective activation of caspase-3 and induction of *IFNB* expression in response to influenza virus.
Fig. S6. Survival and weight loss of ASK1$^{+/+}$ and ASK1$^{-/-}$ mice after influenza infection.
Fig. S7. ASK2 selectively activates an apoptotic response to infection with EMCV.
Fig. S8. ASK2 selectively activates an apoptotic response to infection with influenza virus.

Fig. S9. Overexpression of ASK2 selectively promotes the activation of caspase-3 in response to infection by EMCV.
Fig. S10. Schematic overview of the antiviral strategies mediated by ASK family members.
Fig. S11. Stable association between ASK1 and ASK2 before and after transfection of cells with poly(I:C).

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Fig. 1. The RLR pathway is necessary for effective activation of the p38 and JNK pathways. (**A** to **C**) HeLa S3 cells were transfected with shRNA expression vectors targeting GFP (control), MDA5, RIG-I, or IPS-1 and were cultured for 4 days. The cells were then (A) transfected with the indicated concentrations of poly(I:C) and incubated for 6 hours, (B) infected (ifx) with increasing titers of EMCV for 3 hours, or (C) infected with increasing titers of NDV for 9 hours. Cells were then lysed and subjected to Western blotting analysis with antibodies specific for the indicated proteins. Black vertical bars indicate noncontiguous blots. Western blots are representative of at least three independent experiments.

Fig. 2. TRAFs mediate the IPS-1–dependent activation of the p38 and JNK pathways. (**A**) Scheme showing wild-type (WT) and the indicated mutant IPS-1 proteins. (**B**) HeLa S3 cells were transiently transfected with empty plasmid (vector) or with plasmids encoding WT IPS-1 or the indicated TRAF-binding mutants of IPS-1 (100 or 500 ng, as indicated) together with the PRDIV-Renilla luciferase reporter and pGL3-control as an internal control. The cells were then lysed and luciferase assays were performed. Data are means ± SD of triplicate wells in a single experiment and are representative of three independent experiments (**C**) HeLa S3 cells were transiently transfected with empty plasmid (vector) or with plasmid encoding the dT2/dT3/dT6 IPS-1 mutant and were cultured 24 hours. Cells were then left untreated (0 hour) or were transfected with poly(I:C) (0.25 µg/ml) and then incubated for the

indicated times. The amounts of *IFNB* mRNA were determined by qRT-PCR analysis and are expressed relative to *GAPDH* mRNA. Data are means ± SD of triplicate samples in a single experiment and are representative of three independent experiments ND, not detected. $*P < 0.05$. (**D**) HeLa S3 cells expressing the indicated shRNAs as described in Fig. 1A were left untreated or were transfected with the indicated concentrations of poly(I:C) and incubated for 6 hours. Cell lysates were then subjected to Western blotting analysis with antibodies specific for the indicated proteins. Western blots are representative of three independent experiments.

Fig. 3. ASK1 is activated in response to viral infection and poly(I:C). (**A** to **C**) HeLa S3 cells were (A) transfected with the indicated concentrations of poly(I:C) and incubated for 3 hours, (B) infected with increasing titers of EMCV for 3 hours, or (C) transfected with control vector or plasmid encoding Flag-IPS-1. The cells were then lysed and subjected to Western blotting analysis with antibodies specific for the indicated proteins. H_2O_2 was used as a positive control to activate ASK1. (**D**) HeLa S3 cells were transfected with poly(I:C) and incubated for the indicated times before being lysed and subjected to immunoprecipitation (IP) with anti-ASK1 antibody or control immunoglobulin G (IgG), followed by Western blotting analysis with antibodies against ASK1 and IPS-1. As a control, total cell lysates were analyzed by Western blotting with antibodies against the indicated proteins. All Western blots are representative of three independent experiments.

Fig. 4. ASK1 plays a role in inducing *IFNB* expression and activation of the p38 and JNK pathways. (**A** to **C**) HeLa S3 cells expressing the indicated shRNAs as described in Fig. 1A were transfected with the indicated concentrations of poly(I:C) and incubated for 6 hours (A, left, B, and C), infected with increasing titers of EMCV for 3 hours (A, middle, C), or infected with increasing titers of NDV for 9 hours (A, right, C) before being subjected to (A

and B) Western blotting analysis with antibodies against the indicated proteins and (C) qRT-PCR analysis to determine the abundance of *IFNB* mRNA relative to that of *GAPDH* mRNA. (A, middle) The asterisk indicates a nonspecific band. (B) Whole-cell extracts were analyzed by native PAGE to examine IκBα degradation and IRF3 dimerization. (**D** to **F**) ASK1$^{+/+}$ and ASK1$^{-/-}$ MEFs were transfected with poly(I:C) and incubated for the indicated times or were infected with increasing titers of NDV for 9 hours (D and E), or were infected with higher titer of NDV used in 4E for 24 hours (F). (D) Cell lysates were subjected to Western blotting analysis with antibodies specific for the indicated proteins. (E and F) The relative amounts of *IFNB* and *GAPDH* mRNAs and of NDV APMV1 RNA were determined by qRT-PCR analysis. (**G**) ASK1$^{+/+}$ and ASK1$^{-/-}$ MEFs were infected with SeV-GFP (10^3 p.f.u.) and then were analyzed by immunofluorescence microscopy. Scale bar: 200 μm. Data in (C, left) and (F) are means ± SEM of three independent experiments or ± SD of triplicate samples in a single experiment and are representative of three independent experiments (A, B, C middle and right, D, E, G). *$P < 0.05$, ***$P < 0.005$. Black vertical lines in (A) and (D) denote noncontiguous Western blots.

Fig. 5. ASK1 is required for apoptosis induced by poly(I:C). (**A**) HeLa S3 cells expressing the indicated shRNAs were left untreated (control) or were transfected with poly(I:C) (2.5 μg/ml) and then incubated for 6 hours. Left: Cells were stained with Hochest33342 and analyzed by microscopy. White arrowheads indicate apoptotic cells with pyknotic nuclei Right: The percentages of cells with pyknotic nuclei were determined. Data are means ± SD of values obtained from three fields of 150 to 200 cells (about 600 cells in total) in each of three independent experiments. Scale bar: 300 μm. (**B**) HeLa S3 cells were treated as described in (A) and apoptotic cells were stained with AnnexinV. Data are presented as the fold change in AnnexinV-positive cells relative to untreated control cells. Data are means ±

SEM of four independent experiments. (**C** to **E**) HeLa S3 cells expressing the indicated shRNAs were (C) transfected with the indicated concentrations of poly(I:C) and incubated for 6 hours, (D) infected with NDV for 9 hours, or (E) infected with EMCV for 12 hours. Cells were then lysed and subjected to Western blotting analysis with antibodies specific for the indicated proteins. Data in (A) and (C) to (E) are representative of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Fig. 6 ASK1 mediates antiviral responses to influenza virus in vivo. (**A** to **C**) ASK1$^{+/+}$ and ASK1$^{-/-}$ mice were intranasally inoculated with PBS (Control) or 3.2×10^2 p.f.u. (50 μl) of influenza virus CA04. (A) The amounts of *IFNB* mRNA relative to that of *GAPDH* mRNA in the lungs of the indicated mice were determined by qRT-PCR analysis 1 day after infection. Data are means ± SEM of four independent experiments (from 9 to 11 mice per group). (B) Left: Influenza virus–infected cells in the lungs of the indicated mice were analyzed 3 days after infection by immunohistochemical staining for cleaved caspase-3 (red) and the viral M1 protein (green). The numbers of cells in the bronchioles that were positive for both active caspase-3 and M1 (white arrowheads) were counted. Right: The percentages of infected cells with active caspase-3 were determined. Data in the bar graph are means ± SD of values obtained from 3 to 8 fields of 150 to 200 cells per mouse in each of four independent experiments (about 900 cells in total). (Control: $n = 5$ mice; Influenza: $n = 7$ mice). Scale bar: 10 μm. Note that although the anti-M1 antibody showed a nonspecific signal in uninfected (control) lungs, as indicated by asterisks, bronchiolar epithelial cells were labeled by the anti-M1 antibody only after influenza infection. (C) The viral titers in lungs 3 days after infection were determined by plaque assays in MDCK cells. Data are means ± SEM of four independent experiments. ($n = 15$ to 16 mice per group). (**D**) Age- and sex-matched ASK1$^{+/+}$ mice (n = 17 mice) and ASK1$^{-/-}$ mice (n = 12 mice) were inoculated intraperitoneally with

1×10^5 pfu of EMCV and then were monitored daily for 9 days for survival. Data were analyzed by log-rank test. (**E**) Age- and sex-matched ASK1$^{+/+}$ mice (n = 16 mice) and ASK1$^{-/-}$ mice (n = 11 mice) were infected intraperitoneally with 1×10^2 pfu of EMCV. Two days after infection, the mice were sacrificed, and viral titers in the hearts were determined by standard plaque assay in MEFs from IFN (Alpha, Beta And Omega) Receptor 1 (IFNAR1)$^{-/-}$ mice. Data are pooled from four independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

Fig. 7 ASK2 selectively activates an apoptotic response and inhibits viral replication. (**A** to **D**) HeLa S3 cells expressing the indicated shRNAs were transfected with poly(I:C) and incubated for 6 hours. The amounts of (A) *ASK2* and (B) *IFNB* mRNAs relative to that of *GAPDH* mRNA were determined by qRT-PCR analysis. (C and D) The extent of apoptosis was determined as described in Fig. 5, A and B. Scale bar: 300 μm. (**F** and **G**) 293-ASK2/1KN cells were left untreated or were pretreated with the indicated concentrations of tetracycline (to induce expression of ASK2) and then were transfected with poly(I:C) (7.5 μg/ml) (left) or infected with NDV for the indicated times (right). Data are means ± SEM of four independent experiments (D), or means ± SD of triplicate samples in a single experiment and are representative of three independent experiments (A, B, C, E, F). (**H** to **J**) ASK2$^{+/+}$ and ASK2$^{-/-}$ mice were intranasally inoculated with PBS or 3.2×10^2 p.f.u. (50 μl) of influenza virus CA04. (H) The amounts of *IFNB* mRNA relative to that of *GAPDH* mRNA in the lungs of the indicated mice were determined by qRT-PCR analysis 1 day after infection. Data are means ± SEM of four independent experiments (from 11 to 14 mice per group). (I) Left: Influenza virus–infected cells in the lung were analyzed 3 days after infection by immunohistochemical staining for cleaved caspase-3 (red) and the viral M1 protein (green). The numbers of cells in the bronchioles that were positive for both active caspase-3 and M1

(white arrowheads) were counted. Right: The percentages of infected cells with active caspase-3 were determined. Data in the bar garphs are means ± SD of values obtained from 3 to 8 fields of 150 to 200 cells per mouse in each of three independent experiments (about 900 cells in total). (Control: n = 3 mice; Influenza: n = 5 to 6 mice). Scale bar: 10 μm. (J) The viral titers in the lungs of the indicated 3 days after infection were determined by plaque assays in MDCK cells. Data are means ± SEM of three independent experiments. (n = 10 to 11 mice per group). $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

Figure 1

A

Control shRNA
MDA5 shRNA
IPS-1 shRNA
poly(I:C) :
μg/ml 0 0.25 2.5 0 0.25 2.5 0 0.25 2.5
p-ATF-2
p-c-Jun
p-IRF3
MDA5
IPS-1
p38

B

Control shRNA
MDA5 shRNA
IPS-1 shRNA
EMCV ifx :
p-ATF-2
p-c-Jun
p38

C

Control shRNA
RIG-I shRNA
IPS-1 shRNA
NDV ifx :
p-p38
p-JNK
p-IRF3
RIG-I
IPS-1
p38

Figure 2

A

IPS-1
10 CARD-like domain 71 103 Proline rich region 173 514 TM 535
^{143}PVQETQAPESPGENSE^{158}

dT2 (Q145N)
CARD-like domain Proline rich region TM
^{143}PVNETQAPESPGENSE^{158}

dT3 (T147I)
CARD-like domain Proline rich region TM
^{143}PVQEIQAPESPGENSE^{158}

dT6 (E155D,E457D)
CARD-like domain Proline rich region TM
^{143}PVQETQAPESPGDNSE^{158}
E457D

dT2/dT3/dT6 (Q145N/T147I /E155D,E457D)
CARD-like domain Proline rich region TM
^{143}PVNEIQAPESPGDNSE^{158}
E457D

B

100 ng
500 ng
PRD4 (ATF-2/c-Jun) Luc (fold induction)
0 5 10 15 20 25 30
Vector
IPS-1 WT
IPS-1 dT2
IPS-1 dT3
IPS-1 dT6
IPS-1 dT2/dT3/dT6

C

IFNB mRNA relative expression
0 1.2 2.4 3.6 4.8
*
poly(I:C)(h) : 0 6 9 0 6 9
Vector
IPS-1 dT2/dT3/dT6

D

Control shRNA
TRAF2 shRNA
TRAF6 shRNA
TRAF2 shRNA + TRAF6 shRNA
poly(I:C) : μg/ml 0 0.25 2.5 0 0.25 2.5 0 0.25 2.5 0 0.25 2.5
p-p38
p-JNK
α-tubulin

Figure 3

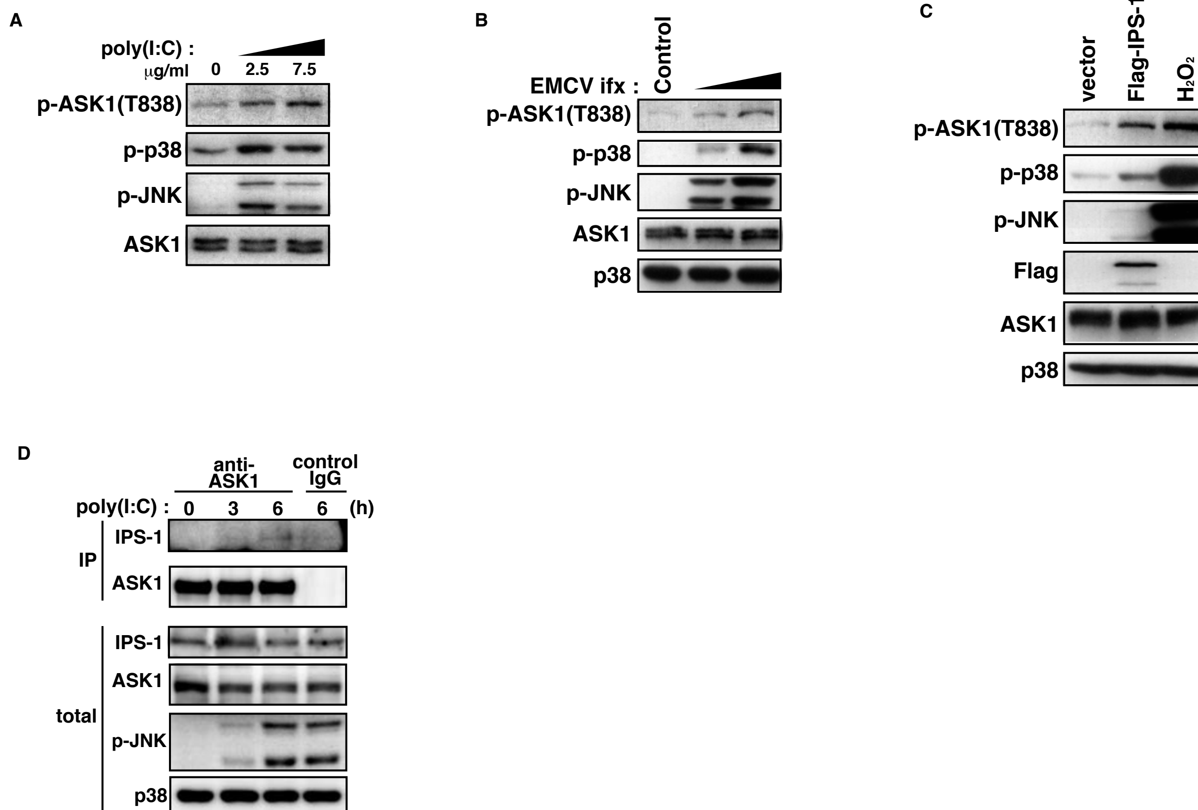
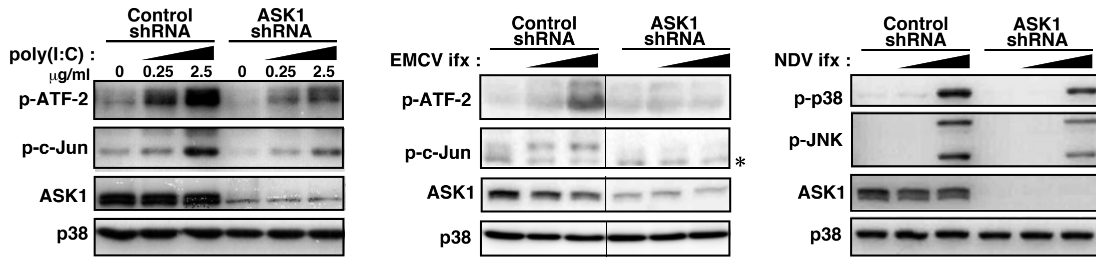
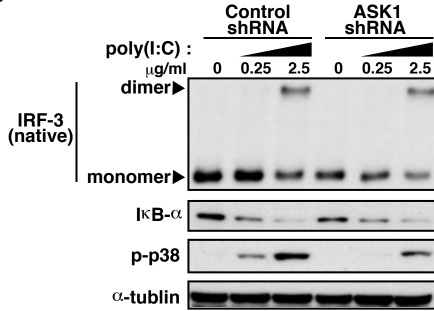


Figure 4

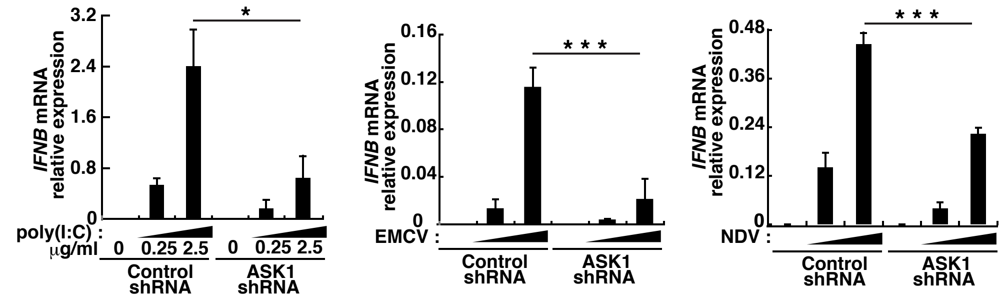
A



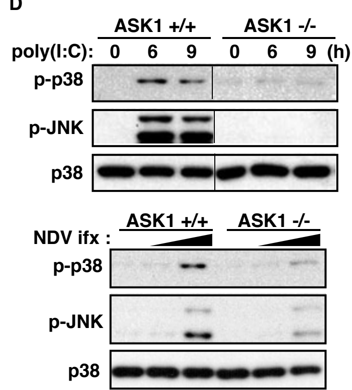
B



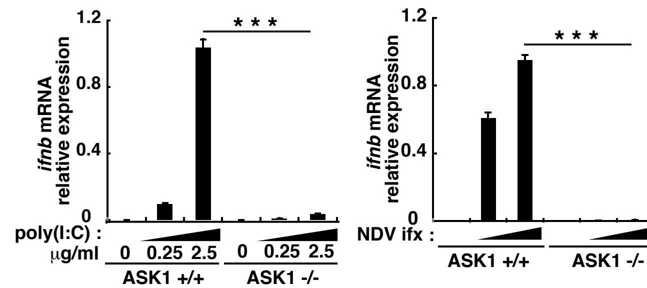
C



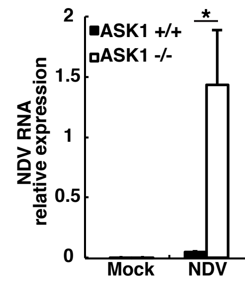
D



E



F



G

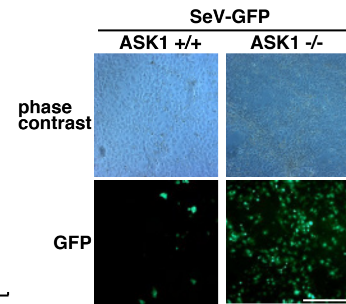
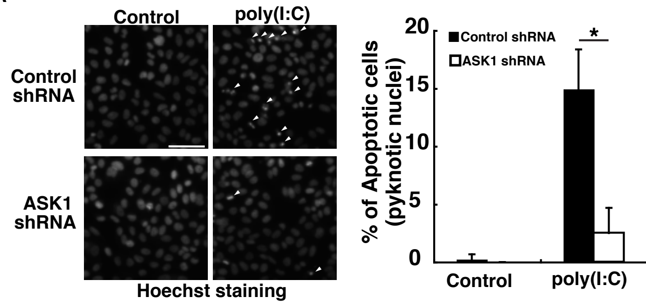
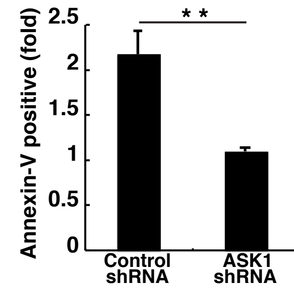


Figure 5





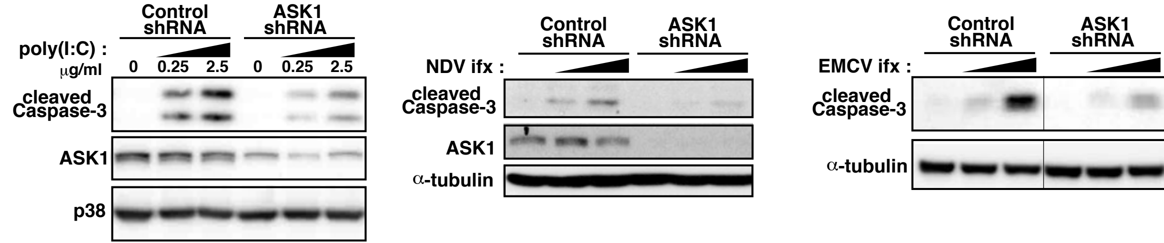


Figure 6

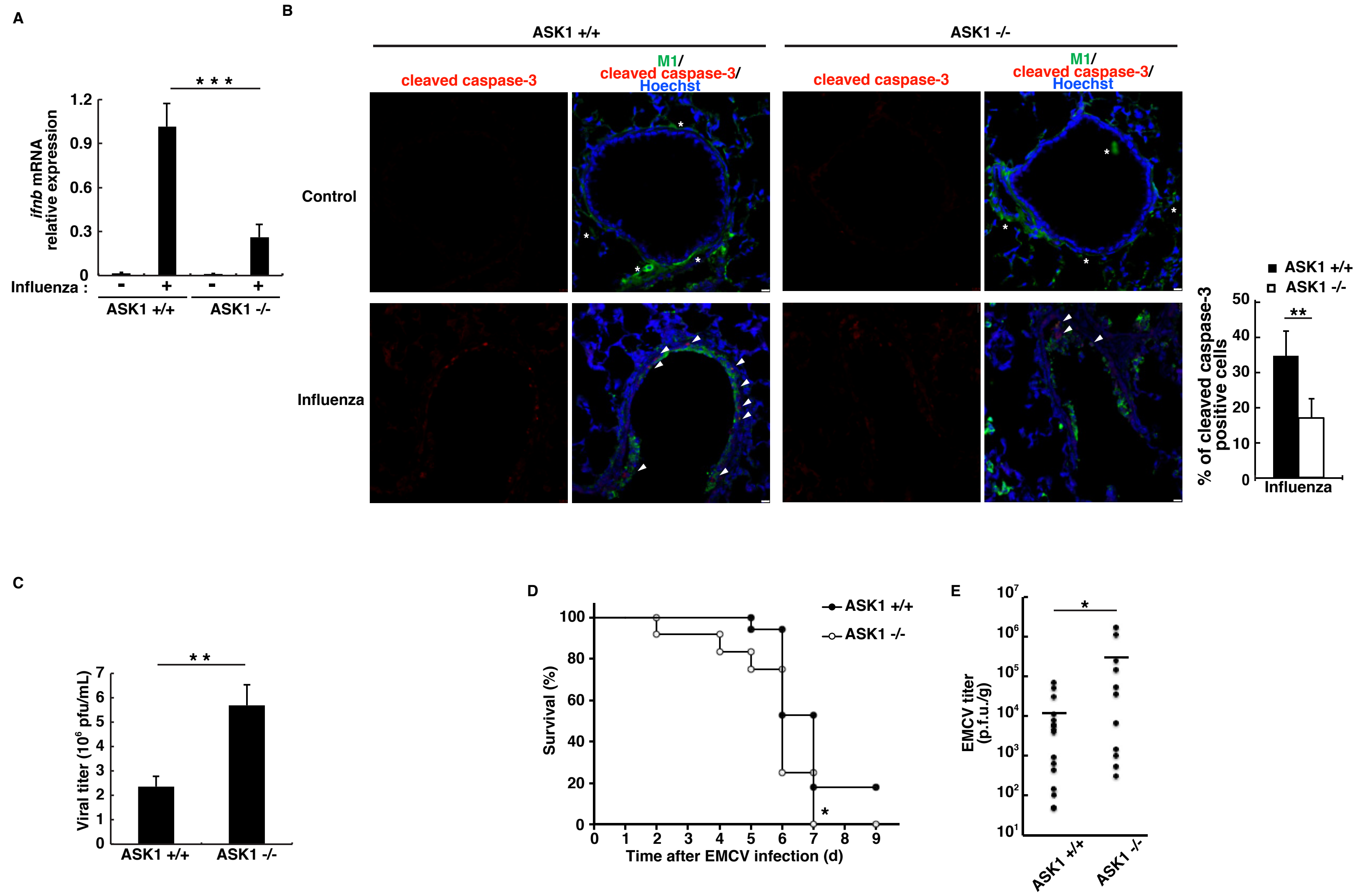
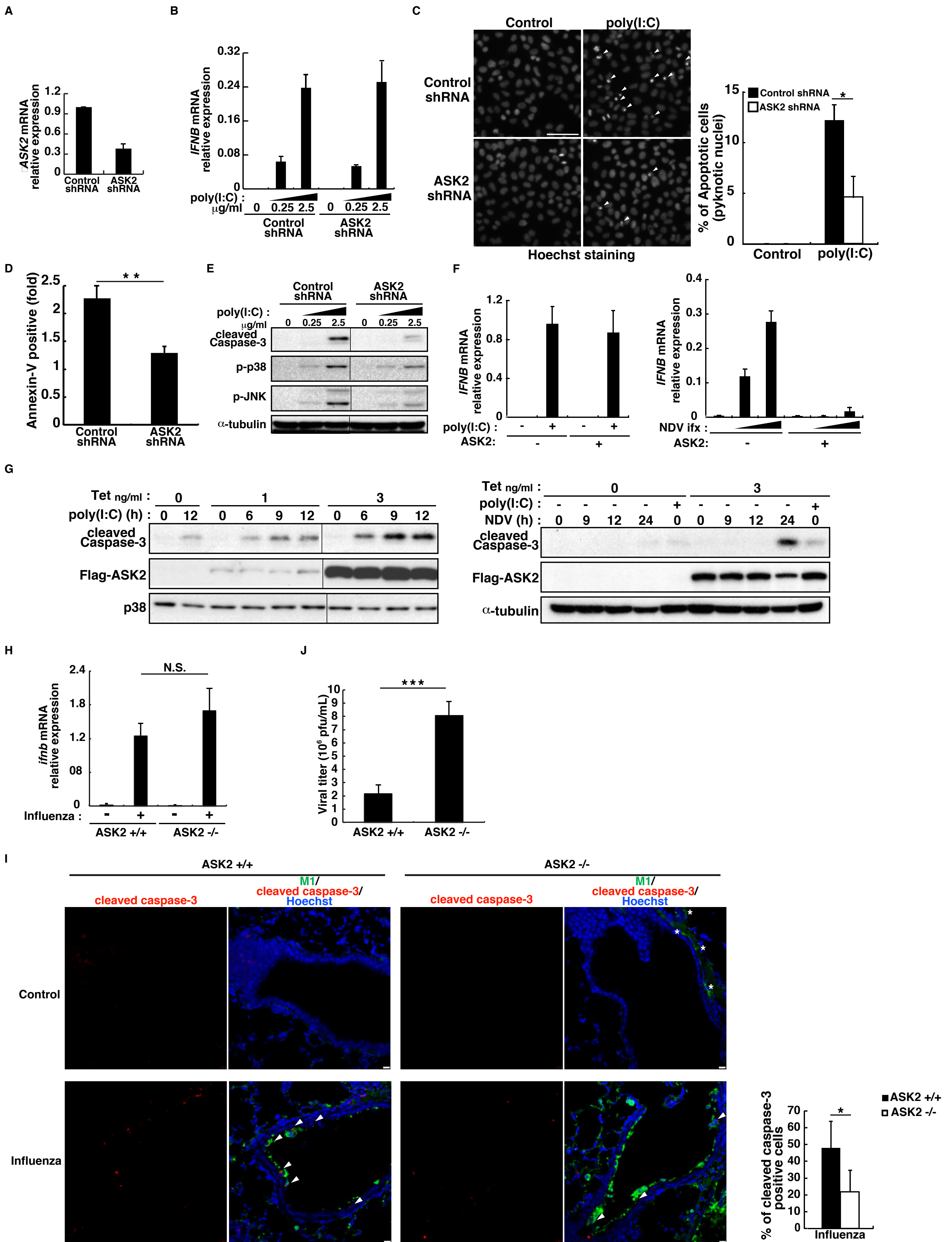


Figure 7



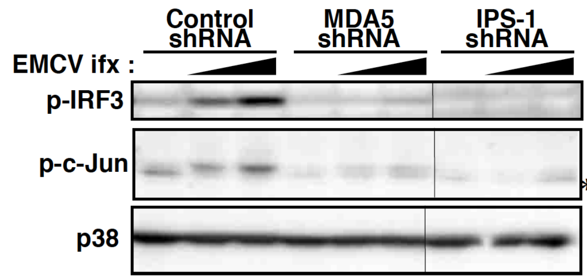


Fig. S1. The RLR pathway is necessary for the activation of IRF3 after infection with EMCV. HeLa S3 cells were transfected with plasmids encoding the indicated shRNAs and cultured for 4 days. The cells were then infected with increasing titers of EMCV for 3 hours. Cell lysates were then subjected to Western blotting analysis with antibodies specific for the indicated proteins. Western blots are representative of three independent experiments. Asterisk indicates a nonspecific band; vertical lines indicate noncontiguous Western blots.

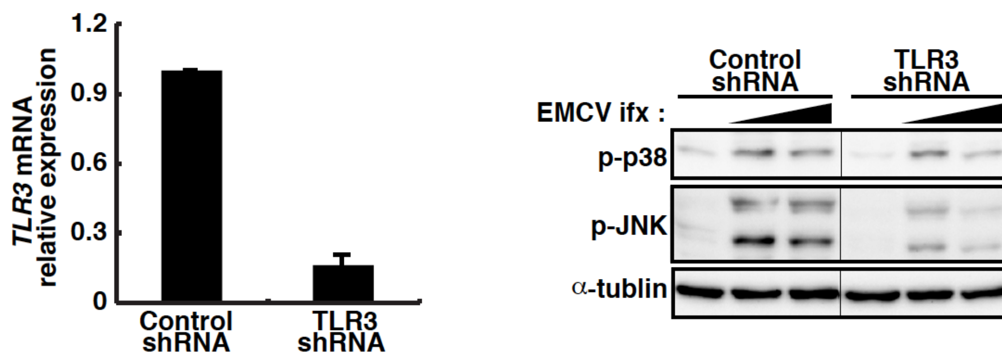


Fig. S2. TLR3 is necessary for the effective activation of the p38 and JNK pathways after infection with EMCV. HeLa S3 cells were transfected with plasmids encoding GFP-specific (Control) or TLR3-specific shRNAs and cultured for 4 days. The cells were then infected with EMCV for 9 hours. Left: The amounts of TLR3 mRNA relative to that of *GAPDH* mRNA were determined by qRT-PCR analysis. Data are means ± SD of three independent experiments and are representative of three independent experiments. Right: Cell lysates were subjected to Western blotting analysis with antibodies specific for the indicated proteins. Blots are representative of three independent experiments.

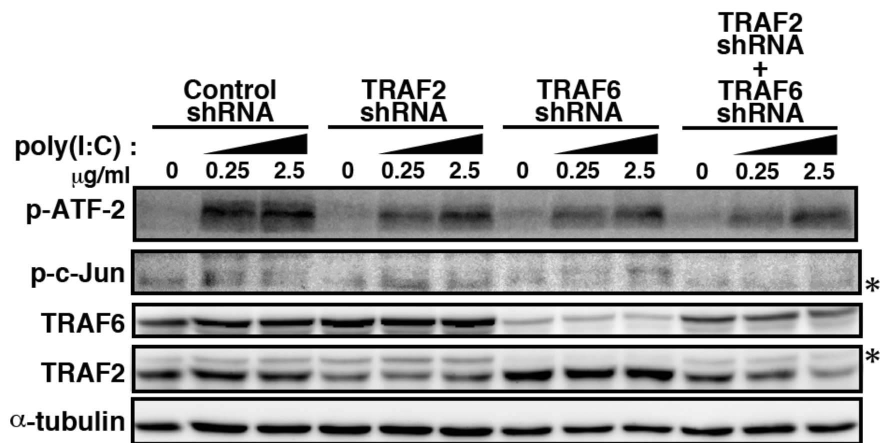


Fig. S2. TRAF2 and TRAF6 mediate the activation of ATF-2 and c-Jun in response to poly(I:C). HeLa S3 cells expressing the indicated shRNAs were transfected with the indicated concentrations of poly(I:C) and incubated for 6 hours. Cell lysates were then subjected to Western blotting analysis with antibodies specific for the indicated proteins. Blots are representative of three independent experiments. Asterisks indicate nonspecific bands.

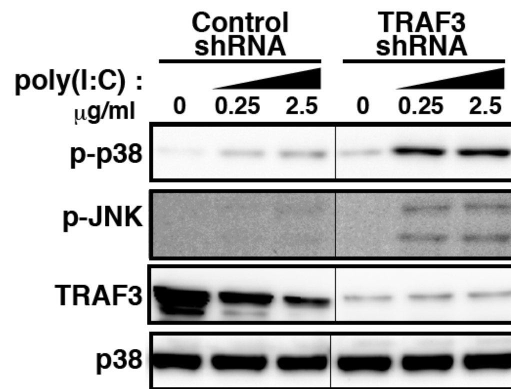


Fig. S4. Analysis of the effect of TRAF3 knockdown on the activation of the p38 and JNK pathways. HeLa S3 cells expressing the indicated shRNAs were transfected with the indicated concentrations of poly(I:C) and incubated for 6 hours. Cell lysates were subjected to Western blotting analysis with antibodies specific for the indicated proteins. Blots are representative of three independent experiments. Vertical lines indicate noncontiguous blots.

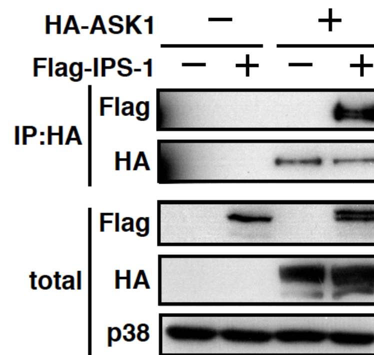


Fig. S5. ASK1 interacts with IPS-1. HEK 293T cells were transiently transfected with the indicated combinations of plasmids encoding HA-ASK1 and Flag-IPS-1. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody, and samples were then analyzed by Western blotting with anti-Flag and anti-HA antibodies. As a control, whole-cell lysates (total) were analyzed by Western blotting with antibodies against the indicated targets. Blots are representative of three independent experiments.

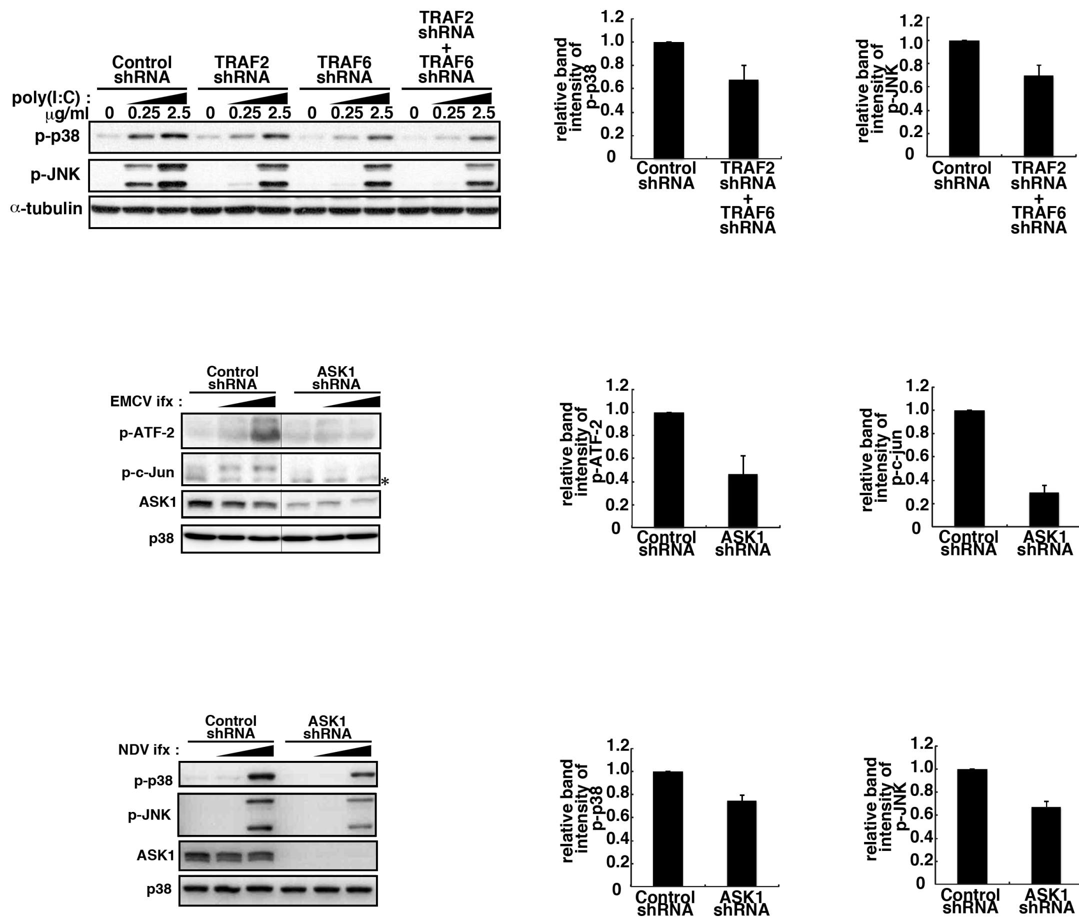


Fig. S3. The quantification of several western blots presented in the manuscript. The intensities were quantified by ImageJ software. Data are presented as relative band intensities of indicated proteins derived from cells with highest doses of stimuli. Data are means ± SEM of three independent experiments.

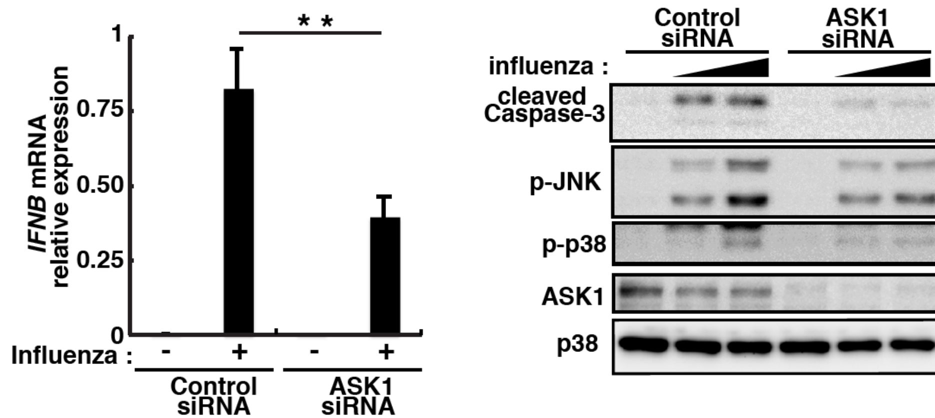
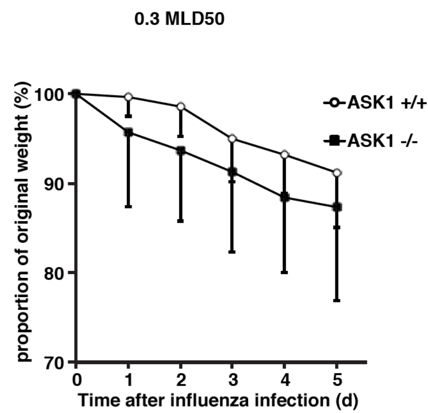
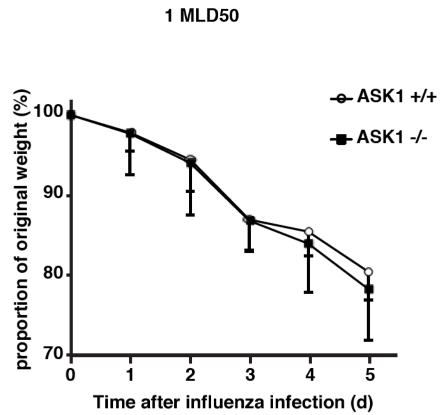


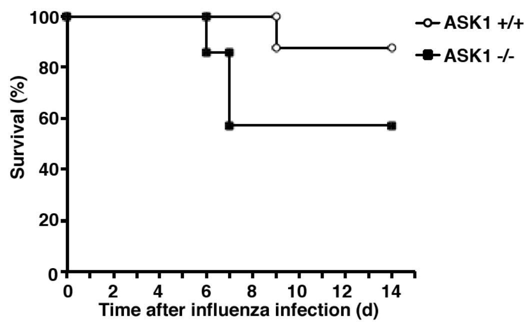
Fig. S5. ASK1 is necessary for effective activation of caspase-3 and induction of *IFNB* expression in response to influenza virus. A549 cells transfected with the indicated siRNAs were left uninfected or were infected with influenza virus (at an MOI of 1 or 10) for 24 hours. Left: The amounts of *IFNB* mRNA relative to that of *GAPDH* mRNA were determined by qRT-PCR analysis. Data are means ± SD of triplicate samples in a single experiment (left) and are representative of three independent experiments. Right: Cell lysates were subjected to Western blotting analysis with antibodies specific for the indicated proteins. Blots are representative of three independent experiments.

A



B





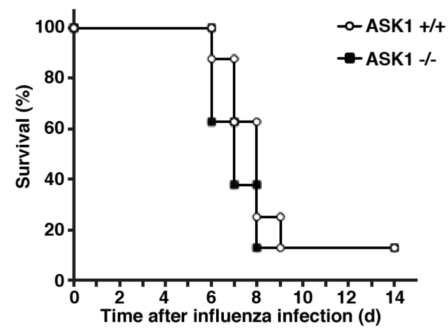


Fig. S6. Survival and weight loss of ASK1$^{+/+}$ and ASK1$^{-/-}$ mice after infection with influenza virus. (**A** and **B**) ASK1$^{+/+}$ and ASK1$^{-/-}$ mice were intranasally inoculated with (A) 1.1×10^2 p.f.u. [ASK1$^{+/+}$ mice (n = 8) and ASK1$^{-/-}$ mice (n = 7)] or (B) 3.2×10^2 p.f.u. [ASK1$^{+/+}$ mice (n = 8) and ASK1$^{-/-}$ mice (n = 8)] of influenza virus. The survival of the mice and changes in their body weight were monitored daily for 14 days.

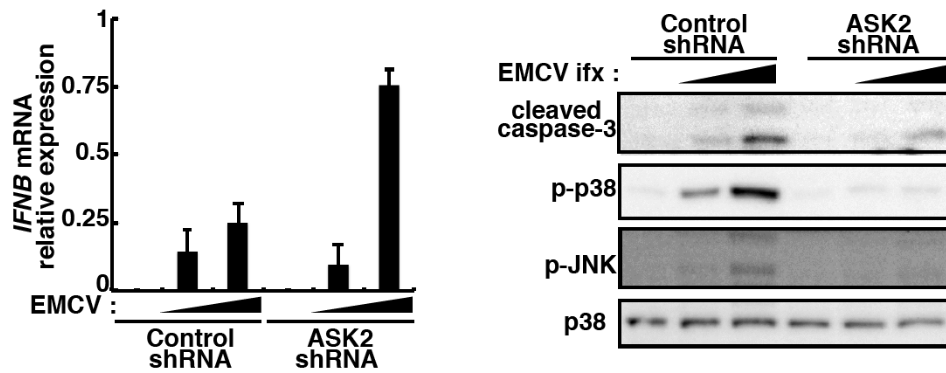


Fig. S7. ASK2 selectively activates an apoptotic response to infection with EMCV. HeLa S3 cells expressing the indicated shRNAs were infected with increasing titers of EMCV for 9 hours. Left: The amounts of *IFNB* mRNA relative to those of *GAPDH* mRNA were determined by qRT-PCR analysis. Data are means ± SD of triplicate samples in a single experiment (left) and are representative of three independent experiments. Right: Cell lysates were subjected to Western blotting analysis with antibodies specific for the indicated proteins. Blots are representative of three independent experiments.

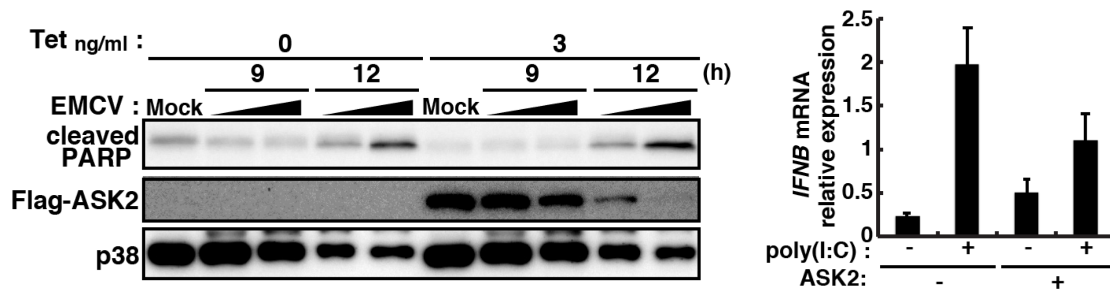


Fig. S9. Overexpression of ASK2 selectively promotes the activation of caspase-3 in response to infection by EMCV. 293-ASK2/1KN cells were left untreated or were pretreated with tetracyclin before being subjected to mock infection or infection with increasing titers of EMCV for the indicated times. Left: Cell lysates were subjected to Western blotting analysis with antibodies specific for the indicated proteins. Blots are representative of three independent experiments. The amounts of *IFNB* mRNA relative to those of *GAPDH* mRNA were determined by qRT-PCR analysis. Data are means ± SEM of three independent experiments.

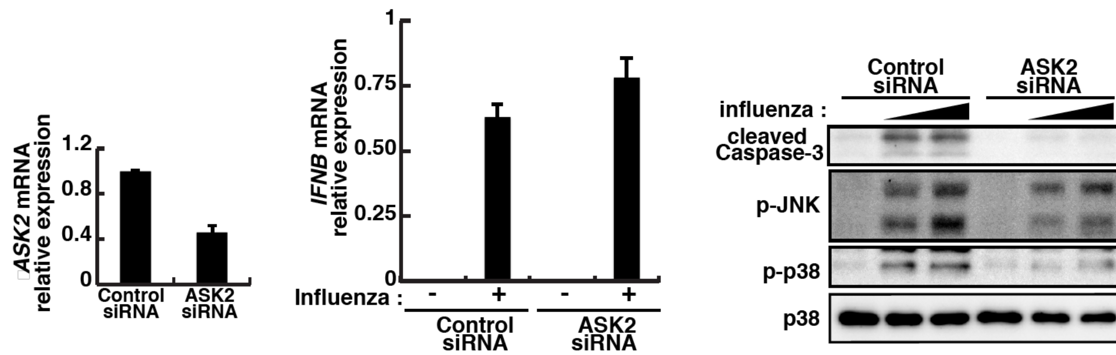


Fig. S8. ASK2 selectively activates an apoptotic response to infection with influenza virus. A549 cells treated with the indicated siRNAs were left uninfected or were infected with influenza virus for 24 hours (at MOIs of 1 or 10). The amounts of *ASK2* (left) and *IFNB* (middle) mRNAs relative to that of *GAPDH* mRNA were determined by qRT-PCR analysis. Data are means ±s.d. of triplicate samples in a single experiment (left, middle) and are representative of three independent experiments. Right: Cell lysates were subjected to Western blotting analysis with antibodies specific for the indicated proteins. Blots are representative of three independent experiments.

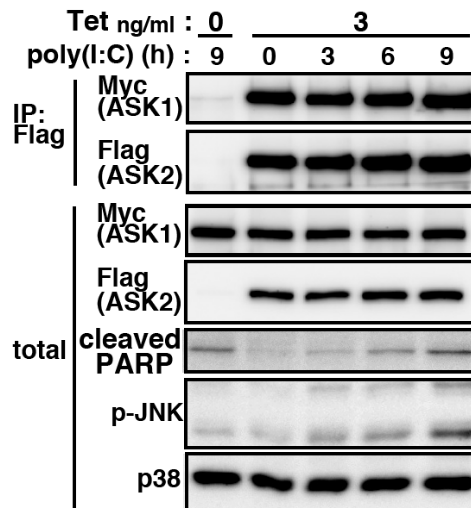


Fig. S11. Stable association between ASK1 and ASK2 before and after transfection of cells with poly(I:C). 293-ASK2/1KN cells were untreated or were pretreated with tetracyclin before being left untransfected or transfected with poly(I:C) (0.25 μg/ml) and then incubated for the indicated times. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag antibody, followed by Western blotting analysis with anti-Flag and anti-Myc antibodies. As a control, whole-cell lysates (total) were analyzed by Western blotting with antibodies against the indicated targets. Western blots are representative of four independent experiments.

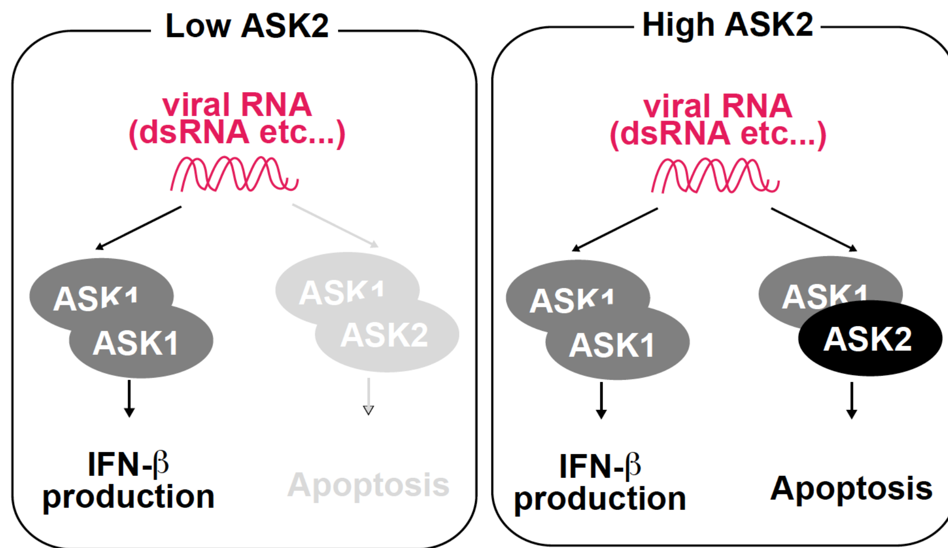


Fig. S10. Schematic overview of the antiviral strategies mediated by ASK family members. See the main text for details.