IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

ASK Family Kinases Are Required for Optimal NLRP3 Inflammasome Priming

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Activation of the multimeric inflammasome complex leads to inflammatory responses to biotic and abiotic triggers. The inflammasome sensor, Nod-like receptor family pyrin domain containing 3 (NLRP3), is activated by a range of stimuli and is tightly regulated to restrict excessive inflammation. Because NLRP3 responds broadly to cellular insults and regulates cell death similar to the stress-activated apoptosis signal-regulating kinases 1 and 2 (ASK1/2), we hypothesized that ASK1/2 may regulate NLRP3 activity. Although essential for mediating NLRP3 inflammasome activation, ASK1/2 were not required for NLRC4 or absent in melanoma 2 inflammasome activation. ASK1/2 was required for NLRP3 up-regulation after lipopolysaccharide treatment in primary bone marrow–derived macrophages and lung fibroblasts as well as during infection with *Burkholderia thailandensis* and influenza virus. Consistent with reduced NLRP3 expression in response to *B. thailandensis*, caspase-1 cleavage and cell death were reduced in infected bone marrow–derived macrophages, and mice lacking ASK1/2 were resistant to *Burkholderia* intranasal infection. Single knockouts of either ASK1 or ASK2 showed a partial role for both ASK1 and ASK2 in NLRP3 up-regulation in response to lipopolysaccharide or *B. thailandensis*, but ASK2 was required primarily to mediate lethal pathology during intranasal infection *in vivo*. Our findings identify the ASK1/2 complex as a regulator of NLRP3 activation and highlight a larger role for ASK2 in lung infection during *B. thailandensis* infection. (*Am J Pathol* 2018, 188: 1021–1030; <https://doi.org/10.1016/j.ajpath.2017.12.006>)

Mitogen-activated protein kinases (MAPKs) are Ser/Thr kinases that are involved in relaying extracellular signals to regulate survival, apoptosis, metabolism, inflammation, and many other cellular processes.¹ Conventional MAPKs include the p38 isoforms, c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), and the extracellular signal–regulated kinases 1/2. These mitogen-activated protein kinase (MAPK)s are regulated by a series of upstream kinases (MAP2K and MAP3Ks) that also are evolutionarily conserved Ser/Thr kinases.² As downstream targets of proinflammatory Toll-like receptor 4 (TLR4), MAP3Ks, including transforming growth factor- β –activated kinase 1 (alias MAP3K7) and apoptosis-signal regulating kinase 1 (ASK1, alias MAP3K5), phosphorylate downstream MKK4/7 and MKK3/6, followed by JNK and p38 MAPK activation.² Although transforming growth factor- β –activated kinase 1 once was considered essential to activate JNK and p38 *in vitro* downstream of TLR activation, recent studies have suggested that this is not universally true in myeloid cells.³ Indeed, ASK1 also

regulates activation of JNK and p38 downstream of TLRs, emphasizing the importance of understanding how each of these MAP3Ks regulates innate immunity.

The ASK family of MAP3Ks, ASK1, ASK2, and ASK3 (encoded by *Map3k5*, *Map3k6*, and *Map3k15*, respectively) are related by sequence homology and interact to regulate the activity of one another.^{4–6} ASK1 is the most studied family member and is an upstream kinase of the JNK and p38 pathways.⁷ Diverse stimuli lead to ASK1 activation including TLR signaling, reactive oxygen species, cytokines, and endoplasmic reticulum stress, and activation is inhibited by thioredoxin and 14-3-3 or promoted by TNF receptor associated factor 2/6.^{8,9} ASK1 drives apoptotic cell death in response to tumor necrosis factor and H₂O₂, which

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can be regulated by ASK2 in certain circumstances, but its role in other forms of regulated cell death are not well studied.^{6,7,10,11} ASK1 and ASK2 also are important in regulating inflammation and cell death during influenza infection, but their role in bacterial infection models is not known, despite the well-established role of ASK1 in the TLR4 signaling pathway.^{12–14}

The inflammasome is a multimeric protein complex that activates caspase-1 and leads to an inflammatory cell death termed *pyroptosis*.^{15,16} The assembly of the inflammasome also involves an adaptor protein ASC [apoptosis-associated speck-like protein containing a caspase activation and recruitment domains (CARD)] containing a CARD and pyrin domain that facilitates the oligomerization of the various sensor proteins Nod-like receptor family pyrin domain containing 3 (NLRP3), NLR family CARD domain containing 4 (NLRC4), and absent in melanoma 2 (AIM2).¹⁶ NLRP3 is the most well-studied inflammasome sensor and NLRP3-mediated inflammasome activation is downstream of a multitude of triggers that are reminiscent of the stimuli activating ASK1, including infectious agents such as influenza virus or bacteria.^{17,18} We therefore hypothesized that the MAP3Ks ASK1 and ASK2 may regulate NLRP3 inflammasome activation in response to intracellular bacterial infection.

ASK1 and ASK2 contribute to the activation of the NLRP3 inflammasome but not the NLRC4 or AIM2 inflammasomes. ASK1 and ASK2 are required to enhance expression of NLRP3 in response to TLR4 stimulation by lipopolysaccharide (LPS), influenza virus, and *Burkholderia thailandensis*. ASK1/2-dependent NLRP3 inflammasome priming also is required for caspase-1 activation during *B. thailandensis* infection of bone marrow–derived macrophages (BMDMs) and causes lethal inflammation in an intranasal infection model. ASK1 and ASK2 both contribute partially to the NLRP3 priming in BMDMs treated with LPS or *B. thailandensis*, but in the intranasal model ASK2 predominantly drives lethal inflammation during infection, consistent with its higher expression in the lung. Together, these data show that in addition to its well-known role in apoptosis, the ASK1/2 signaling complex contributes to pyroptotic cell death by regulating the NLRP3 inflammasome.

Materials and Methods

Mice

Wild-type (WT) (C57BL/6J), *Ask1*^{−/−} (*Map3k5*^{tm1Hijo}),⁷ *Ask2*^{−/−} (*Map3k6*^{tm1Hijo}),¹¹ or *Ask1/2*^{−/−} (*Map3k5*^{tm1Hijo}/*Map3k6*^{tm1Hijo}) mice were obtained from the RIKEN Bio-Resources Center (Tsukuba, Japan) and *Nlrp3*^{−/−}, *Aim2*^{−/−}, *Nlr4*^{−/−}, and *Casp11*^{−/−} have been described previously.¹⁹ Male and female mice between 6 and 8 weeks old were used in this study. Mice were bred at St. Jude Children's Research Hospital and studies were conducted under protocols approved by St. Jude Children's Research Hospital on the Use and Care of Animals.

Bone Marrow–Derived Macrophages

Primary BMDMs were grown for 6 days in Dulbecco's modified Eagle medium (DMEM; 11995073; ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; TMS-013-B; Millipore, Billerica, MA; or S1620; BioWest, Riverside, MO), 30% L929 conditioned media, and 1% penicillin and streptomycin (15070-063; ThermoFisher Scientific). BMDMs were seeded in antibiotic-free media at a concentration of 1×10^6 cells onto 12-well plates and incubated overnight. Cells then were washed and cultured in antibiotic-free DMEM with 10% FBS before stimulations or infections.

In Vitro Isolation and Culture of Lung Fibroblasts

Lung fibroblasts were prepared from the mouse lungs as described previously.²⁰ Briefly, the lungs were harvested, cut into small pieces, and suspended in trypsin lysis buffer 0.25% (25200-056; ThermoFisher Scientific) for 30 minutes at 37°C under agitation. Lung fibroblast medium (DMEM, 10% FBS, 1% penicillin-streptomycin, 1% nonessential amino acid, 1% sodium pyruvate, 1% HEPES buffer, 1% L-glutamine, and 0.25% β-mercaptoethanol) was added to the suspension. The cell suspensions were centrifuged at $300 \times g$ for 5 minutes and pellets were resuspended in lung fibroblast medium and then cultured on tissue-culture–treated dishes. Lung fibroblasts (2×10^5) were seeded onto 12-well cell culture plates in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and stimulated as described for BMDMs.

Bacterial Culture

B. thailandensis strain E264 provided by Dr. Joseph Mougous (University of Washington, Seattle, WA) and *Salmonella enterica* Typhimurium SL1344 were cultured in Luria Bertani broth (3002-031; MP Biomedicals, Santa Ana, CA) overnight and subcultured into fresh Luria Bertani media for 3 hours at 37°C to generate log-phase grown bacteria.

Mouse Infections

B. thailandensis was grown as described above (*Bacterial Culture*). For survival experiments, quantified frozen aliquots of *B. thailandensis* were diluted in phosphate-buffered saline before infection to inoculate at 5×10^5 bacteria per mouse. Mice were anesthetized with isoflurane and administered *B. thailandensis* in a 50-μL phosphate-buffered saline suspension to the nares. After 2 days, lungs and spleens were harvested for histology or colony forming unit analysis. Colony forming units were determined by homogenizing tissue in phosphate-buffered saline with metal beads for 2 minutes using the Qiagen TissueLyser II apparatus (Qiagen, Venlo, Netherlands) and plating on Luria Bertani agar plates incubated overnight.

Bone Marrow—Derived Macrophage Stimulations

BMDMs were differentiated as described above (*Bone Marrow-Derived Macrophages*). Before stimulation, cells were rinsed with phosphate-buffered saline and replaced with fresh antibiotic-free DMEM containing 10% FBS. For inflammasome activation, the following conditions were used to stimulate BMDMs: ultrapure LPS from *Salmonella minnesota* R595 (tlrl-smlps; InvivoGen, San Diego, CA, at 50 ng/mL for 3 hours or transfected at 1 µg/mL for 20 hours), ATP (10127531001; Roche, Basel, Switzerland 5 mmol/L for 30 minutes), *S. enterica* subspecies Typhimurium (multiplicity of infection, 1 for 4 hours), poly dA:dT [poly(deoxyadenylic-deoxythymidylic) acid sodium salt; tlrl-patn; InvivoGen, transfected at 2.5 µg/mL for 4 hours], influenza A/Puerto Rico/8/34 virus PR8 [influenza A virus strain A/Puerto Rico/8/1934 H1N1; multiplicity of infection, 25 in FBS/L-glutamine/sodium pyruvate-free DMEM (D5671; Sigma, St. Louis, MO) for 2 hours, then addition of FBS to 10% until 20 hours], and *B. thailandensis* (multiplicity of infection, 10 for 20 hours). For transfection experiments, LPS and DNA were mixed with 0.3 µL Xfect polymer in Xfect reaction buffer (631318; Clontech Laboratories, Mountain View, CA) and incubated for 10 minutes before being added to BMDMs in Opti-MEM (31985-070; ThermoFisher Scientific). *B. thailandensis* was pelleted onto cells at $300 \times g$ for 5 minutes, washed after 1 hour, incubated with DMEM (1000 µg/mL kanamycin) for 1 hour, washed, and finally incubated in DMEM (250 µg/mL kanamycin) for the remainder of the experiment.

Immunoblotting Analysis

For measuring caspase-1 cleavage, BMDMs and supernatant were collected together in buffer containing NP-40, dithiothreitol, protease inhibitor, and Laemmli sample buffer. For signaling blots, supernatant was removed and cells were lysed in RIPA buffer containing protease and phosphatase inhibitors and Laemmli buffer. Proteins were separated via SDS-PAGE, with 6% to 12% polyacrylamide gels and polyvinylidene difluoride membranes (IPVH00010; Millipore), and blocked with 5% skim milk. Primary antibodies against caspase-1 (AG-20B-0042; Adipogen, San Diego, CA), NLRP3 (AG-20B-00140C100; Adipogen), IL-1β (D4T2D; Cell Signaling Technologies, Danvers, MA), and β-actin (#8457; Cell Signaling Technologies) were incubated overnight at 4°C followed by appropriate secondary antibodies conjugated with horseradish peroxidase incubated for 1 hour at room temperature (Jackson ImmunoResearch, West Grove, PA). Membranes were visualized using Luminata Forte Chemiluminescence substrate (WBLUF0500; Millipore).

RT-PCR Analysis

RNA was isolated using TRIzol (15596026; ThermoFisher Scientific) and converted into cDNA using the High-Capacity

cDNA Reverse Transcription kit (4369914; Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Real-time quantitative PCR was performed on an ABI 7500 real-time PCR machine (Applied Biosystems) with 2× SYBR Green (4368706; Applied Biosystems). Primer sequences for NLRP3 (forward: 5'-TGCAGAAGACTGAC-GTCTCC-3' and reverse: 5'-CGTACAGGCAGTAGAACAGTTC-3') and IL-1β (forward: 5'-GACCTTCCAGGAT-GAGGACA-3' and reverse: 5'-AGCTCATATGGGTCCG-ACAG-3') were used.

Cytokine Analysis

Cytokine levels were determined using an IL-18 enzyme-linked immunosorbent assay (BMS618/3TEN; ThermoFisher Scientific) or multiplex enzyme-linked immunosorbent assay (MCYTOMAG-70K; Millipore) according to the manufacturer's instructions.

Quantification and Statistical Analysis

GraphPad Prism 6.0 software (La Jolla, CA) was used for data analysis. Data are shown as means ± SEM. Statistical significance was determined by the Mann-Whitney test for two groups or one-way analysis of variance (with the Dunnett multiple comparison tests) for three or more groups. Survival curves were compared using the log-rank test. $P < 0.05$ was considered statistically significant. Data from independent experiments were pooled where appropriate.

Results

ASK1/2 Are Required for NLRP3 Inflammasome Activation

Previous work on the ASK1/2 complex focused primarily on the role of downstream signaling on regulating apoptosis in cells exposed to cytokine, endoplasmic reticulum, or infectious or oxidative stressors.²¹ To examine the role of these kinases in inflammasome activation, primary BMDMs were subjected to activators of the canonical NLRP3, NLRC4, and AIM2 inflammasomes, and the noncanonical caspase-11/NLRP3 inflammasome. The canonical NLRP3 inflammasome is activated by treatment with LPS for 3 hours followed by ATP.^{22,23} Upon priming of BMDMs with LPS followed by 30 minutes of ATP treatment, *Ask1/2*^{-/-} macrophages showed reduced caspase-1 cleavage and release of IL-1β and lactate dehydrogenase compared with WT (Figure 1, A and B). This suggests that ASK1/2 signaling is required for potentiation of NLRP3 inflammasome activation.

The NLRC4 inflammasome is activated by ligand binding to NLR family apoptosis inhibitory proteins, with bacterial type III secretion system needle proteins binding NLR family apoptosis inhibitory protein 1, type III secretion system rod protein binding NLR family apoptosis inhibitory protein 2, and flagellin binding NLR family apoptosis

Figure 1 Apoptosis-signal regulating kinases 1/2 (ASK1/2) are required for activation of the Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in bone marrow-derived macrophages (BMDMs). **A:** Immunoblot analysis for caspase-1 cleavage (full-length Casp1 and Casp1 p20) in WT or knockout BMDMs after 50 ng/mL lipopolysaccharide (LPS) and 5 mmol/L ATP stimulation (same blot was cut to remove lanes). **B:** Supernatants were collected and release of lactate dehydrogenase (LDH) and IL-1 β was performed. **C–E:** BMDMs were infected with *S. enterica* subspecies Typhimurium (multiplicity of infection, 1) (**C**), transfected with poly (dA:dT) (**D**), or transfected with LPS (**E**), and caspase-1 cleavage was measured by immunoblot, and IL-18 or IL-1 β release into the supernatant was measured by enzyme-linked immunosorbent assay. Data are representative from at least three independent experiments with significance determined by one-way analysis of variance compared with wild-type (WT) with the Dunn multiple comparison test. * $P < 0.05$, ** $P < 0.005$, and **** $P < 0.0001$ versus WT.

inhibitory proteins 5/6.²⁴ WT and *Ask1/2*^{-/-} BMDMs were infected with *Salmonella* and no defect was observed in NLRC4-dependent inflammasome activation or IL-18 release, suggesting ASK1/2 does not regulate the NLRC4 inflammasome (Figure 1C).

Recognition of cytoplasmic double-stranded DNA is mediated by the AIM2 inflammasome.^{25–28} BMDMs were transfected with poly(dA:dT), and no role for ASK1/2 signaling was found in the activation of the AIM2 inflammasome (Figure 1D). These data show that the AIM2 inflammasome does not require ASK1/2 signaling for activation.

In addition to the canonical NLRP3 inflammasome, noncanonical caspase-11/NLRP3 inflammasome activation occurs after intracellular LPS recognition by caspase-11 and downstream cleavage of gasdermin D, which through an unknown mechanism leads to NLRP3-dependent caspase-1 cleavage.^{29,30} Overexpression of ASK1 has been shown to induce the expression of caspase-11, and the noncanonical inflammasome also is primed by toll-like receptor adaptor molecule 1-dependent interferon up-regulation of caspase-11.^{31–33} Given the role of ASK1/2-dependent signaling in influenza and poly(I:C)-mediated interferon production,¹³ BMDMs were transfected with LPS to determine whether ASK1/2-mediated signaling was required for the regulation of the caspase-11/NLRP3 noncanonical inflammasome. No role for ASK1/2-dependent signaling was observed in cleavage of caspase-1 or release of inflammasome-dependent cytokines after LPS transfection (Figure 1E), suggesting that intracellular

LPS sensing by caspase-11 does not require ASK1/2 activity. No defects were observed in cell death after LPS transfection, which occurs upstream of NLRP3 inflammasome activation (Figure 1E). Together, these data suggest the ASK1/2 complex regulates NLRP3 activation for acute time points after LPS stimulation, as in the case of LPS and ATP stimulation, but is not required during the extended LPS treatment used for intracellular LPS detection by caspase-11 and downstream gasdermin D and NLRP3-dependent caspase-1 cleavage.

ASK1/2 Are Required for Up-Regulation of NLRP3 and IL-1 β

The NLRP3 inflammasome is regulated by both a priming step consisting of transcriptional up-regulation and post-translational licensing followed by a second activation step that leads to the oligomerization of NLRP3 with ASC and caspase-1 to form a catalytically active inflammasome complex.³⁴ To determine whether the NLRP3 priming step was regulated by ASK1/2 signaling, BMDMs were primed with LPS to induce the expression of NLRP3 and IL-1 β and it was found that ASK1/2 are required for transcriptional and translational up-regulation of both NLRP3 and IL-1 β (Figure 2, A and B). LPS-dependent up-regulation of NLRP3 in primary lung fibroblasts also required ASK1/2 signaling (Figure 2C). NLRP3 expression also was reduced in BMDMs infected with the Gram-negative intracellular bacterium *B. thailandensis* and mouse-adapted influenza A virus

Figure 2 Apoptosis-signal regulating kinases 1/2 (ASK1/2) are required for up-regulation of Nod-like receptor family pyrin domain containing 3 (NLRP3) and IL-1 β . **A:** Wild-type (WT) and *Ask1/2^{-/-}* bone marrow–derived macrophages (BMDMs) were stimulated with 50 ng/mL lipopolysaccharide (LPS) for the indicated time, and NLRP3, pro-IL-1 β , and β -actin were measured by immunoblot. **B:** Reverse transcription-PCR analysis was used to measure the relative expression of *Nlrp3* and *Il1b* normalized to expression of the β -actin gene after LPS stimulation. **C–E:** Immunoblots for NLRP3, pro-IL-1 β , and β -actin after stimulation with 50 ng/mL LPS in primary lung fibroblasts (**C**), infection of BMDMs with *B. thailandensis* (multiplicity of infection, 10) (**D**), or infection of BMDMs with IAV PR8 (multiplicity of infection, 25) (**E**). $n \geq 3$ independent experiments. * $P < 0.05$ versus WT (t -test with the Holm-Sidak multiple comparisons test). IAV PR8, influenza A virus strain A/Puerto Rico/8/1934 H1N1.

(Figure 2, D and E). These data show that NLRP3 expression is regulated positively by the ASK1/2 signaling complex.

ASK1/2 Are Required for NLRP3 Inflammasome–Driven Pathology during *Burkholderia* Infection

To determine whether ASK1/2 regulate inflammasome activation during *B. thailandensis* infection, BMDMs from WT, *Nlrp3^{-/-}*, *Nlrp4^{-/-}*, and *Ask1/2^{-/-}* mice were infected. After 20 hours of infection, caspase-1 cleavage was measured by immunoblot (Figure 3A), as well as lactate dehydrogenase and IL-1 β release (Figure 3B), and a decrease in NLRP3-dependent inflammasome activation and pyroptosis was observed in *Ask1/2^{-/-}* BMDMs compared with WT. To determine whether ASK1/2 contributes to *B. thailandensis* infection *in vivo*, WT and *Ask1/2^{-/-}* mice were inoculated intranasally with 5×10^5 colony forming units and monitored for survival. Mice lacking ASK1/2 were protected from lethal infection (Figure 3C) and had reduced bacterial numbers in the spleen at day 2 after inoculation, whereas lung colony forming units were not significantly different (Figure 3D). There were several notable quantitative and qualitative differences between WT and *Ask1/2^{-/-}* mice in both the distribution and severity of pulmonary lesions (Figure 3F). Although neutrophils and macrophages comprised the predominant inflammatory cell infiltrates in both groups, the resulting suppurative and pyogranulomatous lesions generally were larger and coalescing in WT mice, and thus affected a much higher percentage of the alveolar parenchyma than the less clearly defined neutrophil infiltrates in the *Ask1/2^{-/-}*

mice (23% versus 6%, respectively) (Figure 3F). The pyogranulomatous lesions in WT mice were scattered randomly through the lung field and had more clearly defined margins than the lesions in *Ask1/2^{-/-}* mice. Inflammatory lesions in WT mice were characterized by a central suppurative core containing abundant degenerating neutrophils, with more epithelioid macrophages mixed with neutrophils toward the margins. In contrast, pulmonary lesions in *Ask1/2^{-/-}* mice consisted almost entirely of viable neutrophils and abundant macrophages. Other prominent lesions in WT mice that were minimal to absent in the *Ask1/2^{-/-}* lungs included marked edema (both alveolar and perivascular) as well as scattered bronchioles plugged with suppurative exudates (Figure 3F). Consistent with this reduced inflammatory response, a reduction in caspase-1 cleavage (Figure 3E) and reduced proinflammatory cytokines IL-1 β , tumor necrosis factor, and IL-6 (Figure 3G) also were observed. These data suggest that proinflammatory signaling and inflammasome activation downstream of ASK1/2 is responsible for the pathology associated with intranasal *B. thailandensis* infection.

ASK2 Contributes to *Burkholderia*-Mediated Pathology *in Vivo*

The individual contribution of ASK1 and ASK2 to NLRP3 inflammasome priming and *in vivo* pathology was determined using BMDMs from *Ask1^{-/-}*, *Ask2^{-/-}*, and *Ask1/2^{-/-}* mice. BMDMs stimulated with LPS showed that both ASK1 and ASK2 contributed partially to NLRP3 up-regulation (Figure 4A), and this lead to partial reductions in caspase-

1 cleavage in single-knockout BMDMs treated with LPS and ATP (Figure 4B). Interestingly, *B. thailandensis*-infected BMDMs have reduced NLRP3 expression and caspase-1 cleavage in *Ask2*^{-/-} and *Ask1/2*^{-/-} BMDMs, but not *Ask1*^{-/-} cells, suggesting other TLRs may signal via ASK1/2 in distinct ways (Figure 4, C and D). Mice challenged intranasally with *B. thailandensis* are protected when ASK2 or both ASK1 and ASK2 are deleted (Figure 4E), but not when ASK1 alone is deleted. By examining baseline tissue expression from the RIKEN FANTOM5 project database,³⁵ ASK2 was found to be very highly expressed in murine lungs compared with ASK1 (Figure 4F). Taken together, the limited role of ASK1 in BMDMs and the lower expression of ASK1 relative to ASK2 in the lung suggest that ASK2 contributes more to respiratory pathology during *B. thailandensis* infection.

Discussion

In this study, the ASK1/2 complex was found to regulate the expression of NLRP3 and downstream inflammasome activation by LPS and bacterial infection using knockout mouse models. Our results suggest that ASK2 plays a predominant role in the pathology associated with intranasal *B. thailandensis* infection independent from ASK1. Recent work by Okazaki et al¹³ showed that ASK1 and ASK2 have distinct functions in regulating inflammatory cytokines and cell death during influenza infection and these findings suggest other infection models will reveal unique interactions between pathogens and regulation of the ASK1/2 complex. Although most previous research focused on the role of ASK1 signaling in apoptosis, this study showed that ASK1/2 can regulate pyroptotic cell death, suggesting it may have additional functions in other cell death scenarios and in different cell types.

Much of the work on the ASK family MAP3Ks has examined the role of ASK1 in stress responses and how this leads to downstream JNK and p38 apoptotic signaling. ASK2 associates in complexes with ASK1 and each molecule regulates the other through distinct mechanisms.^{6,36} In the absence of ASK1, ASK2 is degraded constitutively so it is interesting that *Ask1*^{-/-} do not mirror the phenotypes observed in *Ask2*^{-/-} and *Ask1/2*^{-/-} knockout mice.⁶ In a study by Iriyama et al,¹¹ ASK2 but not ASK1 limited 7,12-

dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate-induced skin tumorigenesis and also was involved in gastrointestinal cancers. The mechanism they proposed suggested that ASK2 acts as a tumor suppressor in epithelial cancer cell lines whereas ASK1 drives cytokine production.

How ASK1 and ASK2 regulate the expression of NLRP3 remains an interesting area of future study. ASK1 has been shown to phosphorylate MKK3/6 and MKK4/7 to engage p38 and JNK signaling, respectively, but downstream targets of ASK2 have not been well characterized. Interestingly, ASK2 phosphorylates ASK1 at Thr845 but ASK1 does not phosphorylate ASK2; instead, binding stabilizes ASK2 and enhances its activity in a phosphorylation-independent manner, suggesting that tissue level differences in ASK2 may regulate the activity of ASK1.⁶ In addition, a third ASK family member, ASK3, also has been identified by sequence homology and interacts with ASK1, suggesting that it also may play a role in ASK1 and ASK2 signaling cascades.^{4,5} Adding to the complexity of these interactions, reciprocal regulation of ASK1 and transforming growth factor- β -activated kinase 1 also was observed with transforming growth factor- β -activated kinase 1 during *Helicobacter pylori* infection and after stimulation with H₂O₂ or tumor necrosis factor-induced apoptosis, suggesting these complexes may regulate NF- κ B or AP-1-dependent up-regulation of NLRP3.^{37,38} It will be interesting to explore whether ASK2 regulates the activity of other MAP3Ks and downstream targets to regulate NLRP3 expression.

In this study, the high mortality of WT mice in comparison with *Ask1/2*^{-/-} mice is most likely owing to the more severe histopathologic lesions in the lungs of WT mice, which reduced the proportion of remaining functional alveoli. It is likely that the increased degeneration and necrosis of neutrophils in the pulmonary lesions of WT mice contributed to the increased influx of inflammatory cell infiltrates, as well as the more extensive pulmonary edema and numerous exudate-plugged airways. In contrast, the neutrophilic infiltrates in *Ask1/2*^{-/-} mice were milder and consisted mostly of viable neutrophils, which would be consistent with a decrease in pyroptosis. The milder infiltrates of viable neutrophils in *Ask1/2*^{-/-} mice were associated with minimal pulmonary edema and bronchiolar plugging, and thus retention of functional peripheral alveoli that were mostly unaffected by inflammatory cell infiltrates or exudates.

Figure 3 Apoptosis-signal regulating kinases 1/2 (ASK1/2) are required for Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome-driven pathology during *Burkholderia* infection. **A:** Immunoblot for caspase-1 cleavage after bone marrow-derived macrophage (BMDM) infection with *B. thailandensis* (multiplicity of infection, 10; 20 hours). **B:** Release of lactate dehydrogenase (LDH) and IL-1 β in the supernatants of BMDMs infected with *B. thailandensis*. **C:** Survival curve from wild-type (WT) and *Ask1/2*^{-/-} mice infected intranasally with 5×10^5 colony forming units (CFU) of *B. thailandensis*. **D:** *B. thailandensis* CFU recovered from homogenized lung or spleen at day 2 after inoculation. **E:** Immunoblot for caspase-1 p10 cleavage in normalized lung homogenates from day 2 after infection. **F:** Hematoxylin and eosin staining of lungs fixed in formalin at day 2 of infection with *B. thailandensis*, with **green lines** indicating total area and **red outlines** indicating the lesion area used for quantitative analysis. **Boxed areas** are shown at higher magnification in the **insets**. **Insets** magnify the image analysis strategy to measure lesion size and total lung area. **G:** Cytokine enzyme-linked immunosorbent assay from normalized lung homogenate supernatants at day 2 after infection. Data are representative from at least three independent experiments, with significance determined by one-way analysis of variance compared with WT with the Dunn multiple comparison test (**B**), by the log-rank test (**C**), or by the Mann-Whitney test (**D**, **F**, and **G**). * $P < 0.05$, ** $P < 0.005$, and **** $P < 0.00005$ versus WT. Original magnification: $\times 2$ (**F**, top row); $\times 10$ (**F**, middle row); $\times 40$ (**F**, bottom row); $\times 4$ (**F**, inset). TNF, tumor necrosis factor; Uninf, uninfected.

Figure 4 Apoptosis-signal regulating kinase 1 (ASK1) and ASK2 regulate Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome priming but ASK2 predominantly contributes during intranasal infection. **A:** Bone marrow–derived macrophages (BMDMs) from wild-type (WT) or knockout mice were stimulated with 50 ng/mL lipopolysaccharide (LPS) and expression of NLRP3 and β-actin were measured by immunoblot at the indicated time points. **B:** Caspase-1 cleavage was measured after stimulation with LPS and ATP. BMDMs from WT or knockout mice were stimulated with *B. thailandensis* (multiplicity of infection, 10) and expression of NLRP3 and β-actin were measured by immunoblot at the indicated time points (**C**) and caspase-1 cleavage was measured (**D**). **E:** Survival curves were generated after intranasal infection of mice with 5×10^5 CFU *B. thailandensis*. **F:** Tissue expression of ASK1 (*Map3k5*) and ASK2 (*Map3k6*) was generated using publicly available data from the RIKEN FANTOM5 project database (<http://fantom.gsc.riken.jp/5>) and EMBL-EBI Expression Atlas (<http://www.ebi.ac.uk/gxa/home>) with expression in transcripts per million (TPM). **E:** Data are representative from at least three independent experiments or pooled, with significance determined by the log-rank test. $n = 24$ WT (**E**); $n = 27$ *Ask1*^{-/-} (**E**); $n = 21$ *Ask2*^{-/-} (**E**); $n = 14$ *Ask1/2*^{-/-} (**E**). *** $P < 0.0005$, **** $P < 0.00005$.

The initial route of inoculation strongly influences the distribution of pulmonary lesions, and intranasal inoculations of pulmonary pathogens generally results in centripetal spread of infection from proximal airways and central bronchi to the lung periphery. However, the diffuse random distribution of pulmonary lesions that was observed in *B. thailandensis* infected mice typically is associated with a hematogenous route of infection. Taken together, these findings suggest that mice inoculated intranasally with *B. thailandensis* develop an early bacteremia with hematogenous spread to the lung.

Comparing the responses to the diverse stimuli that lead to ASK1/2 and NLRP3-dependent cell death, some common themes emerged. Each pathway was activated downstream of TLR4, required the activity of reactive oxygen species, and each was associated with regulatory proteins including thioredoxin interacting protein, heat shock protein 90, and A20.^{8,39–44} Interestingly, receptor interacting serine/threonine kinase 1, which regulates apoptotic, necroptotic, and some pyroptotic pathways, has been shown to regulate ASK1

activity downstream of tumor necrosis factor, suggesting it may link these pathways.^{39,45,46} Understanding where these pathways interact represents potential new targets for regulating inflammatory diseases. Specific inhibitors of ASK1 or ASK2 may be useful for fine-tuning cellular stress responses and limiting overt pathologic inflammation or cell death.

In summary, our data show that the ASK1/2 signaling complex regulates NLRP3 expression and subsequent NLRP3 inflammasome activation downstream of TLR4 and during infection by *B. thailandensis*. The deletion of ASK2 but not ASK1 specifically contributes to limiting inflammatory pathology in the lungs of mice infected with *B. thailandensis*, and ASK1/2 signaling is associated with reduced inflammation, caspase-1 cleavage, and proinflammatory cytokines in the lung. These findings suggest that ASK family kinases may regulate other NLRP3-dependent cell processes in response to different stimuli or in different cellular contexts, contributing to inflammatory disease and cell death processes beyond apoptosis.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.12.006>.

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