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NLRP3 (NALP3, cryopyrin) facilitates *in vivo* caspase-1, necrosis, & HMGB1 release via inflammasome-dependent and –independent pathways[1]

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Abstract

Bacterial infection elicits a range of beneficial as well as detrimental host inflammatory responses. Key among these responses are macrophage/monocyte necrosis, release of the pro-inflammatory factor high-mobility group box 1 protein (HMGB1), and induction of the cytokine IL-1. While the control of IL-1β has been well-studied, processes that control macrophage cell death and HMGB-1 release in animals are poorly understood. This study utilizes *Klebsiella pneumonia* as a model organism since it elicits all three responses *in vivo*. The regulation of these responses is studied in the context of the inflammasome components, NLRP3 and ASC, which are important for caspase-1 activation and IL-1β release. Using a pulmonary infection model that reflects human infection, we show that *K. pneumonia*-induced mouse macrophage necrosis, HMGB-1 and IL-1β release are dependent on NLRP3 and ASC. *K. pneumoniae* infection of mice lacking *Nlrp3* results in decreased lung inflammation and reduced survival relative to control indicating the overall protective role of this gene. Macrophage/monocyte necrosis and HMGB1 release are controlled independently of caspase-1 suggesting that the former two responses are separable from inflammasome-associated functions. These results provide critical *in vivo* validation that the physiologic role of NLRP3 and ASC is not limited to inflammasome formation.

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Introduction

The NLR (nucleotide binding domain, leucine rich repeats-containing) family (formerly known as CATERPILLER, NOD, NACHT-LRR, NOD-like receptor) family of genes/proteins is increasingly implicated in the regulation of immunity (1). The NLR family member NLRP3 (formerly cryopyrin, CIAS1, NALP3), which is expressed abundantly in neutrophils and macrophages, has emerged as a critical mediator of inflammation. *NLRP3/CIAS1*, the human gene encoding this protein, was first identified through its association with the hereditary periodic fever syndrome, CAPS (*CIAS1*-associated periodic syndrome), which comprises a wide range of inflammatory symptoms and severity (2–6). This condition results from gain-of-function mutations in *NLRP3*. Disease-associated variant forms of NLRP3 exhibit enhanced capacity to induce caspase-1 and IL-1β maturation, and a necrotic pathway of macrophage cell death that indirectly enhances inflammation (7–9).

A role for NLRP3 in caspase-1 maturation is well-studied. Following stimulation, NLRP3, the adaptor ASC (Apoptotic Speck-like protein containing a Card), Cardinal/TUCAN, and pro-caspase-1 combine to form one of several known inflammasome complexes. Within this complex, pro-caspase-1 is activated, which in turn cleaves and activates the pyrogenic cytokines IL-1β and IL-18 (9). Currently, the inflammasome protein complex has also been demonstrated for NLRP1 (NALP1), and the function of caspase-1/IL-1β release has been associated with NLRC4 (IPAF/CLAN) and NAIP5, however activation of the NLRP3 inflammasome is associated with the widest spectrum of stimuli. Among these are gram-positive and gram-negative bacteria, including *Staphylococcus aureus, Listeria monocytogenes*, and *Shigella flexneri,* toxins as well as uric acid crystals and pathogen or non-pathogen derived nucleic acid (8, 10, 11). Environmental pollutants such as asbestos and silica, as well as particulate adjuvants and β-amyloid have also been found to require NLRP3 for caspase-1 mediated cytokine secretion (12–16)

Recent work has suggested a second pro-inflammatory function for NLRP3 involving monocyte necrosis (7, 8). Although the study of this process has been previously limited to *in vitro* culture, this form of necrosis has been shown to occur in monocytic cells infected with intracellular bacteria or following exposure to toxins. Both microbial pathogen (*S. flexneri*) induced necrosis and necrotic death associated with CAPS require NLRP3, its partner protein ASC, and the lysosomal protease cathepsin B (8). However, NLRP3-dependent necrosis is entirely independent of caspase-1 and IL-1β, and other apoptosis-associated caspases and has been named pyronecrosis. One defining feature of monocytic necrosis is the loss of plasma membrane integrity and subsequent spilling of intracellular inflammatory contents, most notably the nuclear factor HMGB1, which elicits strong pro-inflammatory effects when released into the microenvironment (17). HMGB1 can activate the RAGE receptor and TLRs to elicit proinflammatory responses including the release of TNF-α and IL-1β. HMGB1 is considered a therapeutic target as antibodies against HMGB1 have been shown to effectively reduce sepsis, arthritis, and cancer in animals (18–20). However NLR-dependent inflammatory necrosis or HMGB1 release remain to be validated in a physiologic setting (21).

Klebsiella pneumoniae is among the most common gram negative bacteria encountered by clinicians worldwide and is a leading cause of community-acquired and hospital-associated respiratory infection (22). Its frequency in the latter context is particularly alarming as *K. pneumoniae* is responsible for up to 23% of nosocomial infections, and a mortality rate of up 50% in elderly or otherwise compromised patients (23). Moreover, the growing prevalence of antibiotic resistant strains in this species has led to increased attention and concern (24, 25). *K. pneumoniae* is a non-motile, non-flagellated, gram negative, rod-shaped bacterium which normally resides within the mouth, skin, and intestines. Pathogenic *K. pneumoniae*

invades the lungs where it is capable of inducing severe bacterial pneumonia that is often complicated with bacteremia and sepsis (26). Airway infection typically leads to extensive lung injury resulting from increased inflammation, hemorrhage, and the necrotic destruction of lung tissue. This process results in thick, blood-laced mucous known as “currant jelly” sputum, which is characteristic of *K. pneumoniae*-induced pneumonia. In addition to cellular necrosis, this bacteria also induces HMGB1 release in humans (27) . Though recent work has started to identify innate immune mechanisms underlying these diverse host responses to bacterial infection, little work has been done to examine the regulation of these diverse inflammatory responses elicited by *K. pneumoniae* infection, or the contribution of these mechanisms to pathogenesis or immunity (28–30). Due to the multiple inflammatory responses that are elicited by *K. pneumoniae* including cytokines, necrosis and HMGB1 induction, and its potential as a public health threat caused by the rise of antibiotic-resistance strains, we elected to study the roles of NLRP3 and ASC using this bacteria in animals.

Despite the wealth of *in vitro* data implicating the NLR family in pathogen-induced inflammation, *in vivo* evidence for the importance of these proteins in inflammation has been limited to IL-1β and IL-18 release. This report shows that NRLP3 activates macrophage necrosis as well as HMGB1 release in addition to IL-1β/IL-18 secretion in response to *K. pnuemoniae* in infected animals. NRLP3 is an important immune protection factor during infection with this bacteria as its absence decreases the inflammatory response and the rate of survival in mice. This is the first *in vivo* analysis depicting an inflammasome-independent function of NRLP3 which reveals a much broader physiologic role for NRLP3 that extends beyond IL-1β/IL-18 secretion.

Materials and Methods

Experimental Animals

All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Mice deficient in *Nlrp3ASC, Nlrc4,* and *caspase-1* were generated as previously described (31, 32). All animals were maintained in pathogen-free facilities at The University of North Carolina at Chapel Hill.

Cell lines and reagents

THP-1 cells were obtained from American Type Culture Collection (ATCC) and cultured as described previously (33). Antibodies: anit-caspase-3 from Cell Signaling; anti-PARP, anti-Actin, and HRP-conjugated secondary antibodies from Santa Cruz Biotechnology; anti-HMGB1 from Abcam; Super Signal ECL reagent from BioRad. The preparation of retroviral vectors and THP-1 cell lines stably expressing shRNA has been described (34). The shRNA target sequences are: shASC-GCTCTTCAGTTTCACACCA, shCtrl-GCTCTTCctggcCACACCA, shNLRP3-GGATGAACCTGTTCCAAAA.

Bacteria

K. pneumoniae 43816, serotype 2 was obtained from the ATCC and cultured in LB. Bacteria density was estimated by measuring the absorbance at 600 nm (1 $OD^{600} = 3 \times 10^8$ bacteria/ml). Accurate CFUs were determined for each experiment by plating an aliquot on LB agar plates.

Bacterial induced inflammation

Cultures of *K. pneumoniae* were pelleted, washed twice in PBS, and resuspended in PBS. Mice were anesthetized and challenged via intratracheal (i.t.) instillation with 7.4×10^4

CFUs of *K. pneumoniae* in 50 μl of PBS, as previously described (28). Mock challenged mice received 50 μl of PBS. THP-1 cells were infected with *Klebsiella* at a MOI=50 for 6 hours. Bone marrow derived macrophages (BMDM) were prepared as previously described (8) and infected with *Klebsiella* (MOI=200, 6 hours) or *Salmonella* (MOI=50, 1 hour). Samples were centrifuged at 650*g for 10 minutes immediately following addition of bacteria. Gentamicin (50 μg/ml) was added to cultures 1 hour post infection.

Assessment of bacteria burden

Mice were euthanized via i.p. injection with 2,2,2 tribromoethanol (avertin). Whole liver, spleen and lungs were removed, wet weight assessed, homogenized in 500 μl HBSS with a Tissue Master 125 (Omni International) and centrifuged. The resulting supernatants were plated on LB agar plates.

Assessments of airway inflammation

Mice were euthanized via i.p. injection of avertin and serum was harvested via cardiac puncture. The liver, kidney, and spleen were removed, weighed, and either homogenized in 500 μl HBSS or fixed in 4% paraformaldehyde (PFA). Mice were then perfused with HBSS and a tracheal cannula was inserted below the larynx. The lungs were lavaged 5 times with 1 ml HBSS followed by centrifugation to isolate BAL cells and cell free supernatants. Red blood cells were lysed via hypotonic saline treatment and cellularity assessed with a hemacytometer. Aliquots of BALF were cytospun onto slides and Diff-Quik (Dade Behring) stained for differential cell counts. Leukocytes were identified based on morphology of ≥200 cells per sample. An aliquot of BALF was plated on LB agar to assess bacteria burden. The remaining BALF was centrifuged and the supernatant was collected. Following BALF harvest, the lungs were fixed by inflation (20-cm pressure) and immersed in 4% PFA.

Histopathologic examination

For histopathological examination, lungs were fixed by inflation (20-cm pressure) and immersion in 10% buffered formalin. Whole inflated lungs were embedded in paraffin wax. Sections (4-μm) were cut and stained with hematoxylin and eosin (H&E). Serial sections of the left lobes of the lungs that yield maximum longitudinal visualization of the intrapulmonary main axial airway were examined and inflammation was scored by one of the authors (I.C.A.) who was blinded to genotype and treatment. Histology images were evaluated and each of the following inflammatory parameters was scored between 0 (absent) and 3 (severe): mononuclear cell infiltration; polymorphonuclear cell infiltration; airway epithelial cell hyperplasia/injury; extravasation; perivascular cuffing; and percent of lung involved with inflammation, as previously described (35). The scores of the parameters were averaged for a total histology score.

Cytokine and chemokine assessment

Cell free supernatants from *K. pneumoniae* (MOI=200, 6 hour) or *S. typhi* (MOI=50, 1 hour) infected thioglycolate-elicited peritoneal macrophages were analyzed using RayBio® Cytokine Antibody Array G Series 3 (RayBiotech Inc). Axon scanner 4000B with GenePix software was used to collect fluorescence intensities. These values were normalized to the ratio of positive control values for each sample. Total normalized fluorescence values of replicate spots were averaged and expressed as fold increase over non-infected samples. “N/D” indicates cytokines where fluorescence values of replicate spots deviated more than 2 fold and were thus dismissed. If this occurred in the non-treated sample, the cytokine was removed from the data set. Cytokine concentrations were determined by RayBiotech Inc. using their Quantibody service.

ELISA assay

Samples were collected at indicated times and assayed with OptEIA Human IL-1β ELISA Set or OptEIA Mouse IL-1β or IL-6 ELISA Sets (BD).

Cell viability determination

Viability was assayed per manufacturer protocol using either CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega), ToxiLight® BioAssay Kit (Lonza Bioscience), or 7-AAD (BD) staining as indicated. In the case of 7-AAD staining, cells were collected, washed twice in PBS, resuspended in 0.5ml PBS with 1 μl 7-AAD, and incubated for 15 minutes before analysis on a FACScan (BD).

Results

K. pneumoniae-induced cell death and IL-1β release require NLRP3 and ASC

The role of NLR proteins in host immune response to *K. pneumoniae* was first examined in a human macrophage cell line. We observed both cell IL-1β release (Fig. 1A) and cell death (Fig. 1B) following infection of THP-1 human monocytic cells with *K. pneumoniae*. Both processes were abrogated in a human macrophage cell line with reduced NLRP3 expression (Fig. 1A and 1B). Reduction of NLRP3 was achieved through stable integration of retroviruses encoding shRNAs designed to promote the targeted degradation of NLRP3 mRNA (labeled shNLRP3), as described and demonstrated previously (8, 34).

Previously, we demonstrated that ASC, a partner protein of NLRP3, is required for *S. flexneri*–induced cell death in THP-1 cells (8). Therefore, we tested the ability of *Klebsiella* to elicit cell death and IL-1β in ASC-deficient THP-1 cells. Both IL-1β release and cell death were substantially abrogated in the shASC cells (Fig. 1C and 1D). Having established that NLRP3 is essential for *Klebsiella*-induced cell death, we sought to determine the nature of this phenomenon. Indicative of pyronecrosis, *K. pneumoniae*-induced cell death was markedly reduced in control THP-1 cells treated with the cathepsin-B inhibitor Ca-074-Me (Fig. 1E). The caspase-1 specific inhibitor YVAD-cho had no effect on cell death as measured by LDH release (Fig. 1E). The caspase-1 specific inhibitor was used at concentrations sufficient to inhibit caspase activity, as evidenced by the attenuation of *Klebsiella*-induced IL-1β release (Fig. 1F). Interestingly, the cathepsin B inhibitor not only blocked *Klebsiella*-induced cell death in THP-1 cells, but also prevented IL-1β release in response to the pathogen (Fig. 1F). This indicates that cathepsin B also controls IL-1β release.

Additional features of *Klebsiella*-induced cell death are also consistent with pyronecrosis. During apoptosis, caspase-3 undergoes activating cleavage. In turn, caspase-3 cleaves PARP and other downstream substrates. Neither caspase-3 nor PARP were cleaved in shCTRL or shNLRP3 cells infected with *Klebsiella*, though both were cleaved in staurosporine-treated cells (Fig. 1G). During macrophage necrosis, HMGB1 is released. In accordance with this, HMGB1 is released from *K. pneumoniae* infected shCTRL cells but not infected shNLRP3 cells as determined by a western blot (Fig. 1H). HMGB1 is not released by THP-1 cells treated with staurosporine, a well-established inducer of apoptotic cell death (Fig 1H). HMGB1 release from shCTRL cells is abrogated by a Cathepsin B inhibitor (Ca-074-Me), indicating that *Klebsiella* induced HMGB1 release requires cell death (Fig 1I).

Klebsiella-induced IL-1β is reduced in *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ macrophages

To examine the physiologic importance of these results, BMDM were isolated from WT mice and mice deficient for *Nlrp3, Asc,* or *Nlrc4*. Deletion of *Nlrp3* or *Asc* resulted in a near complete inhibition of IL-1β induced by *Klebsiella* as measured by ELISA, whereas *Nlrc4*

null macrophages demonstrated no substantial difference from WT (Fig. 2A). Importantly, this phenomenon is not common to all pathogenic bacteria. In agreement with previous work, IL-1β release from macrophages infected with *S. typhi* was unaffected by *Nlrp3* deletion, whereas deletion of *Nlrc4* eliminated the inflammatory response (Fig. 2A). The NLRP3 inflammasome was previously reported to be activated by a combination of *E. coli* lipopolysaccharide (LPS) and ATP (9). To determine if the NLRP3 inflammasome is also activated by *Klebsiella* LPS, BMDM were challenged with 50 ng/ml LPS isolated from *K. pneumoniae* for 16 hours followed by stimulation with 5 mM ATP for 20 minutes. In contrast to WT and *Nlrc4* deficient macrophages, deletion of *Asc* or *Nlrp3* eliminated *Klebsiella* LPS-induced IL-1β release (Fig. 2B). In agreement with observations in THP-1 cells, activation of the inflammasome by *K. pneumoniae* was abrogated by both caspase-1 specific inhibitors (YVAD) and the cathepsin B inhibitor, CA-074-Me (Fig. 2C). Together, these results indicate that NLRP3 is the predominant NLR activated by *Klebsiella* and that deletion of either *Nlrp3* or *Asc* substantially abrogates host inflammatory responses.

K. pneumoniae induces chemotactic and inflammatory cytokine production in primary mouse macrophages

The processing and release of proinflammatory cytokines and chemokines is fundamental to proper innate immune response to pathogens. To more broadly assess the effect of NRLP3 and ASC on host inflammatory responses, cell free supernatants prepared from *Klebsiella*- or *Salmonella*-infected macrophages were analyzed on anti-cytokine antibody arrays containing antibodies to 62 inflammatory mediators. Production of IL-1β was markedly decreased in both *Asc* and *Nlrp3* deficient macrophages as expected, but GM-CSF and IL-1α were also reduced indicating that these cytokines are coordinately controlled by ASC and NRLP3 either directly or indirectly (Fig 3A). In contrast, Mip-1 was coordinately enhanced in *Nlrp3* and *Asc*-deficient macrophages when compared to control cells, perhaps to compensate for the loss of IL-1β. IL-6 and RANTES were increased in the $Nlrp3^{-/-}$ but not $Asc^{-/-}$ cells. As a pathogen-specificity control, IL-1β was not decreased in *Nlrp3* deficient macrophages treated with *Salmonella*, which activates the Nlrc4 inflammasome. To confirm the results obtained by the antibody array, a subset of inflammatory mediators from *K. pneumoniae* challenged *ex vivo* macrophages were measured using quantitative multiplexed anti-cytokine arrays. Cytokine measurements of IL-1β, Mcp-1, and KC in the absence *of Asc* and *Nlrp3* are consistent with the data obtained by the antibody arrays (Fig. 3B, complete data set shown in Table S1).

Mice lacking *Nlrp3* and *Asc* demonstrate significantly increased mortality following *K. pneumoniae* airway infection

Our *in vitro* results suggested that ASC and NRLP3 are critical regulators of *K. pneumoniae* induced inflammation and cell death. To determine if inflammation was also reduced *in vivoAsc* and *Nlrp3* deficient mice were challenged with (7.4×10^4) CFUs of *K. pneumoniae* delivered through airway infection. Previous reports show that E. coli LPS enhances NLRP3 expression *in vitro* (36). To assess this issue in animals in the context of *K. pneumoniae*, we show that *in vivo* NRLP3 expression is greatly induced (600×) by this bacteria while ASC expression is increased by 25× (Fig. 4A). To determine whether NRLP3 and ASC are involved in mediating the overall host response to *K. pneumoniae in vivo*, animals were challenged via intratracheal instillation and survival was assessed over the course of 4 days. Mice lacking the *Nlrp3* gene demonstrated moderate but statistically significant increased mortality compared with WT mice ($p < 0.05$, Logrank Test) (Fig. 4B). *Asc* deficient mice demonstrated similar increases in mortality (Fig. 4C). No significant difference in survival was observed between *Nlrc4*-deficient mice and the WT controls (Fig. 4D). This moderate effect of *Nlrp3* on animal survival may be explained by other NLRs that can mediate inflammasome formation in the lung, as well as by the compensatory increase in other

inflammatory cytokines, such as IL-6, which might mask the effect of *Nlrp3*-deficiency in animals.

Nlrp3 deficient mice demonstrate significantly attenuated airway inflammation following *K. pneumoniae* infection

The above results suggested that NRLP3 is an important mediator of several inflammatory cytokines and is essential for mouse survival in an *in vivo* model of *K. pneumoniae* infection. To determine if airway inflammation was attenuated, *Nlrp3* deficient mice were challenged via intra-tracheal instillation with *K. pneumoniae* and the lungs were harvested 48 hours post inoculation for histology analysis. Lung sections were prepared to reveal the main bronchi of the left lobe from the indicated genotypes and representative sections were examined (10× and 20× magnification) (Fig. 5A). In comparison to WT and *Nlrc4*$^{-/-}$ mice, *Nlrp3*$^{-/-}$ mice show decreased inflammatory cell recruitment and less occlusion of the alveolar spaces (Fig. 5A). These findings are consistent with a significant attenuation in airway inflammation observed in mice lacking *Nlrp3*, but not *Nlrc4*, following pulmonary challenge with *K. pneumoniae* (Fig. 5B).

In vivo levels of IL-1β and cell death are reduced in *K. pneumonia* challenged *Nlrp3*$^{-/-}$ mice

We have demonstrated that NLRP3 is required for necrosis in both human and mouse cell cultures challenged with *Klebsiella*. We further demonstrate that NRLP3 mediates pulmonary inflammation and protects against the bacterial infection. We next sought to assess the *in vivo* relevance of this gene in cytokine production, bacterial-induced necrosis and HMGB1 release. Processes that regulate the latter two have not been extensively studied in an *in vivo* setting. To determine the physiologic effects of NRLP3, we first measured IL-1β levels in the BALF and serum of *K. pneumoniae* infected mice. Deletion of *Nlrp3* caused a near-abrogation of serum IL-1β, which is consistent with the important role NRLP3 plays in IL-1β production by monocytes (Fig. 6A). Deletion of *Asc* also resulted in marked decreases in circulating levels of IL-1β (Fig. 6A). IL-1β in the BALF was modestly reduced in the *Nlrp3*$^{-/-}$ mice, a finding that is similar to other *in vivo* analyses (13). The modest effect is likely due to redundant NLR proteins in the lung stroma that remained intact in these mice. This decrease in IL-1β was accompanied by a modest increase of IL-6 observed between *Nlrp3*$^{-/-}$ and WT mice in serum and BALF samples (Fig. 6C–D), which is consistent with the cytokine array analysis of *in vitro* cultured cells shown in Fig. 3. No significant differences were observed in the cellular composition of the BALF between genotypes (Table S2). Deletion of *Nlrp3* also decreased overall levels of cell death in BALF samples as determined by LDH release (Fig. 6E). To examine whether NRLP3 is responsible for the induction of HMGB1, HMGB1 levels were measured in serum samples of *K.pneumoniae* challenged mice. As measured by western blot analysis, *Klebsiella*-induced HMGB1 release was substantially abrogated in *Nlrp3*$^{-/-}$ mice (Fig. 6F). Having determined that NLRP3 was involved in the initiation of cell death in response to *K. pneumoniae in vivo*, we sought to determine if this cell death was morphologically consistent with necrosis. Cells collected from BALF samples obtained from *K. pneumoniae* infected animals were subjected to electron microscopy analysis. Cells harvested from WT animals demonstrate several morphological features consistent with necrosis, including loss of plasma membrane integrity and lack of chromatin condensation (Fig. 6G). In contrast, cells obtained from *Nlrp3* deficient animals exhibit no signs of cell death, despite the presence of several intracellular *K. pneumoniae* bacteria (Fig 6G). These results indicate that Nlrp3 mediates *K. pneumoniae* induced inflammation and cell death *in vivo*, and that this cell death is morphologically and biochemically consistent with necrosis.

Discussion

Several recent reports have established a role for NRLP3 in mediating pathogen-induced inflammation *in vitro*, but the majority of evidence has been provided using *ex vivo* cultures obtained from gene-deletion mice. Here, we identify NRLP3 as a critical *in vivo* effector of the host immune response to *K. pneumonia*, a major cause of community-acquired bacterial pneumonia. *K. pneumoniae* was selected for analysis because it induces a flagrant cytokine and inflammatory response, including the release of HMGB1, and significant necrosis in the lung, thus allowing us to assess the effect of NRLP3 and ASC on all three processes. This is the first combined *in vitro* and *in vivo* demonstration that an NLR molecule is a key regulator of not only IL-1β maturation, but also HMGB1 release and necrotic cell death in response to pathogen exposure. Furthermore, these results demonstrate the *in vivo* consequences of NRLP3 activity on host survival and inflammation. Despite substantial decreases in lung inflammation and tissue destruction (as evaluated by cytokine analysis and criteria specified in Materials and Methods), mice lacking *Nlrp3* demonstrate increased susceptibility to *Klebsiella*-induced lethality. This finding confirms that NRLP3 activity contributes to protective host responses to bacterial pathogens via both inflammasome-dependent and independent processes. This is similar to recent findings regarding the role of NRLP3 during infection by influenza virus where the *Nlrp3* gene is found to be important for increased pulmonary inflammation, cellular infiltrate and IL-1β/IL-18 production, all of which coincide with increased animal survival and decreased viral load (37, 38).

After *S. flexneriK. pneumoniae* is the second gram negative bacterial pathogen identified which activates the NLRP3-dependent cell death program termed pyronecrosis (8). This pathway of cell death has morphological features characteristic of necrosis, and similar to necrosis is inherently pro-inflammatory. Cellular components spill out from the pyronecrotic cell into the microenvironment. Among these components is HMGB1, a nuclear protein which takes on the role of a powerful pro-inflammatory cytokine when released from the cell (17, 39). HMGB1 stimulates the RAGE, TLR2, and TLR4 receptors on neighboring monocytes and macrophages and results in the induction of several inflammatory cytokines, including TNFα and IL-1β (40–42). *K. pneumoniae* induces a significant increase in the systemic levels of HMGB1 in the WT mice, while no HMGB1 is observed in the serum from *Nlrp3*$^{-/-}$ animals. This NLRP3-dependent release of HMGB1 is also observed in human THP-1 monocytic cells challenged with *Klebsiella*. Caspase-1 inhibitors failed to abrogate NLRP3-mediated HMGB1 release, suggesting that this phenomenon does not require inflammasome activity. It should be noted that HMGB1 levels are significantly increased in human septic patients, including those with *K. pneumoniae* sepsis (27). Neutralization of HMGB1 is currently under investigation as a therapeutic target for the intervention of sepsis, bacteremia, and induced acute respiratory distress syndrome (43, 44). Though a broad inhibition of NLRP3-dependent responses may be detrimental to host survival, neutralization of HMGB1 may provide an opportunity to abrogate NLRP3-mediated sepsis or bacteremia without increasing host mortality.

Pyronecrosis serves as an interesting contrast to pyroptosis, another form of pathogen-induced cell death (45). While pyronecrosis requires cathepsin B but not caspase activity, pyroptosis requires the activity of caspase-1 (21). These two pathways also appear to be induced by different stimuli and involve different NLRs. Pyronecrosis has been observed in monocytic cells infected with 50 MOI of *Shigella flexneri,* an MOI previously shown to cause necrosis (8). Pyroptosis has been observed in monocytic cells infected with *Salmonella* (31, 46), *Pseudomonas aeruginosa* and low dose (<10 MOI) *S. flexneri* (31, 46–48). Interestingly, though ASC is required for activation of caspase-1, deletion of ASC does not abrogate caspase-1 dependent pyroptosis initiated by *P. aeruginosa* (47).

Our *in vitro* and *in vivo* results indicate that NLRP3-dependent pyronecrosis is the predominant cell death and inflammation pathway induced by *Klebsiella*. In contrast to the *Salmonella*-induced pyroptosis pathway which is dependent on caspase-1 and NLRC4, ablation of the *Nlrc4* or *caspase-1* gene has minimal effect on inflammation or cell death induced by *K. pneumoniae*. Interestingly, cathepsin B inhibitors not only block NRLP3-mediated pathogen induced cell death, but also block the induction of IL-1β maturation by *K. pneumoniae*. A similar requirement for cathepsin B in NRLP3 mediated IL-1β maturation in response to silica crystals, aluminum salts, and lysosomal permeabilization has also been recently reported (16, 49). These finding suggests that a significant portion of *K. pneumoniae*-induced IL-1β release might lie downstream of pyronecrosis, which is not surprising given the strong pro-inflammatory activity of HMGB1.

Previously, we demonstrated that NLRP3 and ASC were required for the release of IL-1β and HMGB1 from a human macrophage cell line, THP-1, infected with *Shigella flexneri.* Here we have shown that the release of IL-1β and HMGB1 is also markedly decreased from *Nlrp3* and *Asc* deficient macrophages infected with *K. pneumoniae* and extended this finding to a study of the whole animal. Furthermore, our results reveal additional cytokines and chemokines, such as GM-CSF and IL-1α, which are also reduced in the absence of *Nlrp3* or *Asc*. Perhaps to compensate for the lack of these inflammatory mediators, the levels of Mip-1α, IL-6, and RANTES were increased in $Nlrp3^{-/-}$ or $Asc^{-/-}$ cells. The mechanisms by which NRLP3 and ASC regulate the production of these cytokines and chemokines are currently under investigation, however these results demonstrate that NRLP3 and ASC may directly or indirectly influence the inflammatory response to *K. pneumoniae*, through both inflammasome dependent and independent pathways.

In summary, our results indicate that NLRP3 and ASC are key regulators of necrosis and inflammation associated with *K. pneumoniae* infections in human and mouse cells. Most important, this body of work demonstrates that altering specific NLRs can dramatically affect the *in vivo* host immune response in a common and clinically relevant model of Gram-negative bacterial pneumonia. Given the clear phenotype observed in the *Nlrp3* and *Asc* deficient mice, it will be interesting to further define the mechanisms underlying the attenuation of inflammation and increased mortality, with special emphasis on the role of HMGB1. Our findings suggest the cumulative effect of NRLP3 on cytokines and chemokines, cell death, and HMGB1 protects the host from pathogen induced mortality. These findings exemplify the complex interactions associated with NLRP3 in innate immune signaling and potential limitations of therapeutic intervention of NLRP3 activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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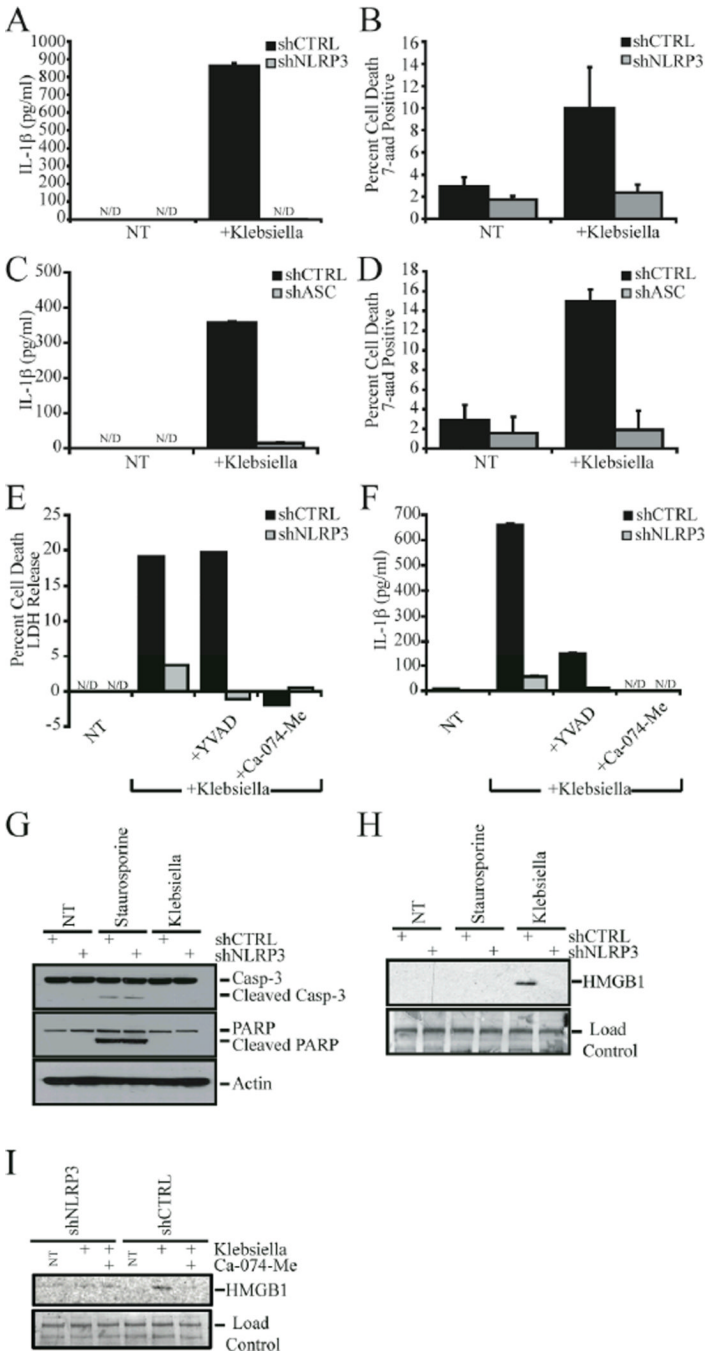

Figure 1. *Klebsiella pneumoniae* induced cell death and IL-1β required NLRP3 and ASC

A) *K. pneumoniae* induced IL-1β release was decreased in THP-1 cells stably transduced with NLRP3-specific shRNA (shNLRP3). B) shNLRP3-THP-1 cells were resistant to *K. pneumoniae*-induced death. C and D) ASC was required for *K. pneumonia*-induced IL-1β release (C) and cell death (D) in THP-1 cells. E and F) Addition of 50 μM cathepsin B inhibitor (Ca-074-Me) substantially abrogated *K. pneumoniae* induced cell death (E) and IL-1β release (F) in shCTRL and shNLRP3 cells. In contrast, 100 μM caspase-1 specific (YVAD-CHO) inhibitors failed to block *K. pneumoniae*-induced cell death (E), but did inhibit IL-1β release (F). G) Caspase-3 and PARP were cleaved in response to an apoptotic stimulus (staurosporine), but not in THP-1 cells following *K. pneumoniae* infection. H) *K.*

pneumoniae-induced HMGB1 release was abrogated in cells deficient in NLRP3 IL-1β release was determined by ELISA. I) Addition of 50 μM cathepsin B inhibitor (Ca-074-Me) substantially abrogated *K. pneumoniae* induced HMGB1 release. Caspase-3, PARP, and HMGB1 levels were assessed by western blot analysis. NT, no treatment; N/D, not detected.

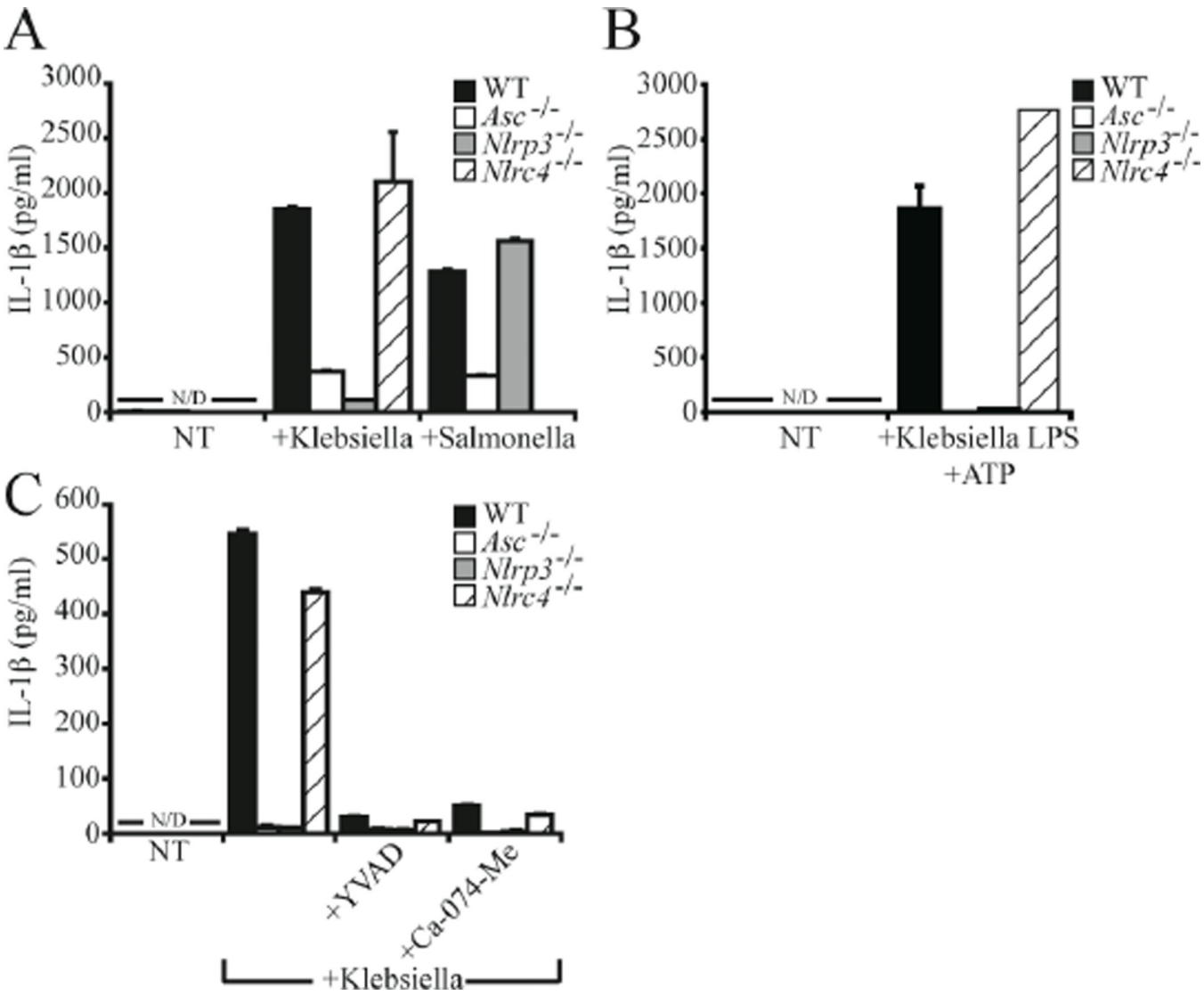

Figure 2. *Klebisella pneumoniae* induced NLRP3 and cathepsin B dependent IL-1β release in primary macrophages

A) *Nlrp3* and *Asc* deficient BMDM exhibited decreased levels of IL-1β release in response to *K. pneumoniae* but not *S. typhi.* Macrophages lacking *Nlrc4* demonstrated no defect in *K. pneumoniae* induced IL-1β release, but IL-1β release was attenuated in response to *S. typhi.* B) *K. pneumoniae* LPS (50 ng/ml, 16 hours) in combination with ATP (5 mM, 20 min.) stimulated NLRP3 and ASC-dependent, but NLRC4-independent, IL-1β release from BMDM. C) Addition of either 100 μM caspase-1 inhibitor (YVAD) or 50 μM cathepsin B inhibitor (Ca-074-Me) substantially abrogated *K. pneumoniae* induced IL-1β release from BMDM. IL-1β was measured by ELISA. NT, no treatment; N/D, none detected.

A

	>5 Fold Induction Over Mock				
	+Klebsiella			+Salmonella	
	WT	*Asc*$^{-/-}$	*Nlrp3*$^{-/-}$	WT	*Nlrp3*$^{-/-}$
MIP-1α	126	264	293	108	80
IL-1α	97	40	31	69	38
MIP-2	85	89	103	80	96
IL-6	82	80	156	19	22
GM-CSF	82	44	29	4	1
TNFα	79	93	93	20	11
MCP-1	58	66	46	20	N/D
KC	55	38	66	49	46
RANTES	26	28	57	21	16
IL-12 p40/p70	14	18	19	2	2
IL-1β	12	1	3	11	10
TIMP1	8	4	5	2	1
IP10	5	4	5	1	1

B

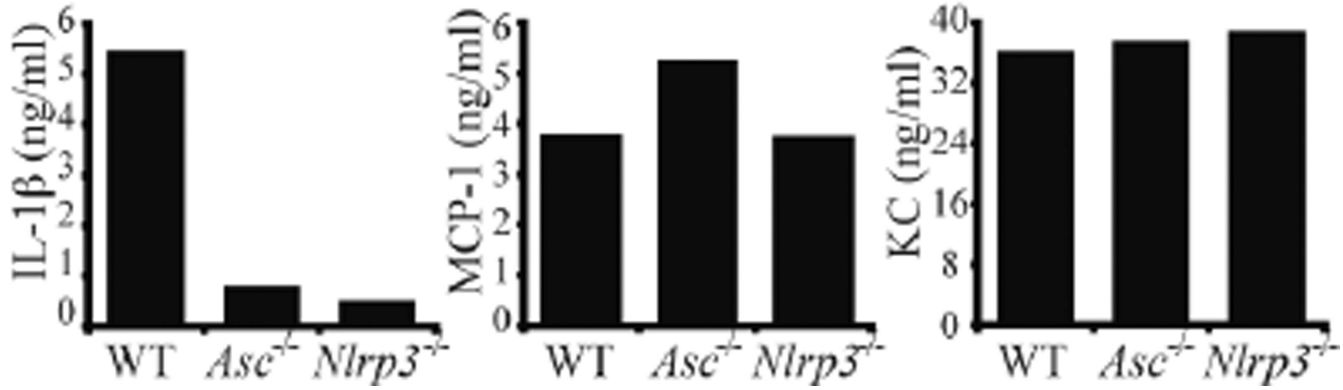

Figure 3. *Klebsiella pneumoniae* induced inflammatory cytokines and chemokines in primary macrophages

A) Thioglycolate-elicted peritoneal macrophages of indicated genotypes were infected with *K. pneumoniae* (MOI=200, 6 hours) or *S. typhi* (MOI=50, 1 hour). Cell free supernatants were analyzed on RayBiotech G Series 3 cytokine antibody arrays. Cytokines and chemokines induced ≥5 fold over the non-treated control of each genotype are shown. B) Quantification of IL-1β, MCP-1, and KC support trends observed on G Series 3 cytokine arrays. Cytokine levels were determined using RayBiotech Quantibody service.

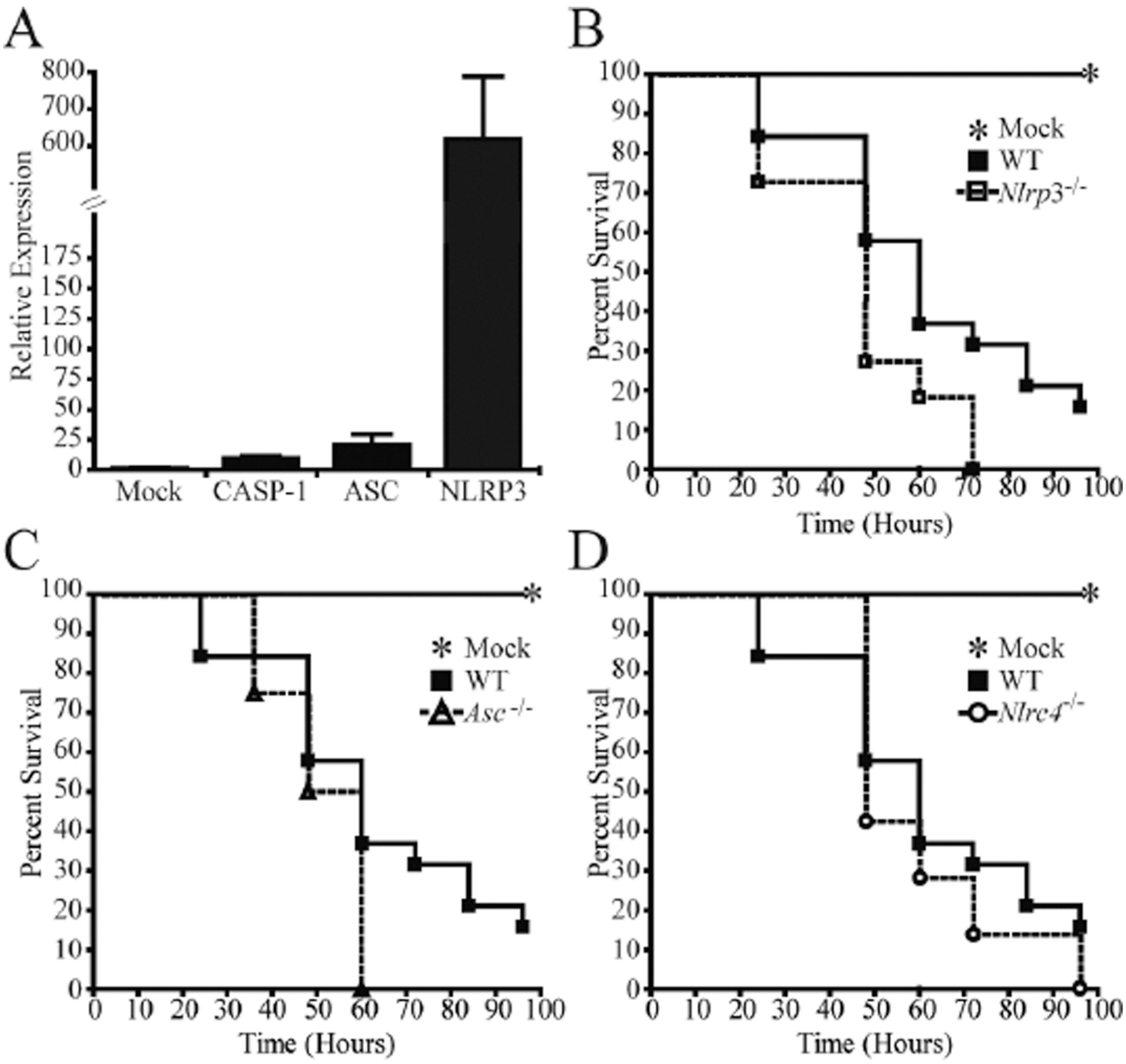

Figure 4. *Nlrp3* and *Asc* deficient mice demonstrated significantly increased mortality following *K. pneumoniae* airway infection

Mice were intratracheally challenged with (7.4×10^4) CFUs of *K. pneumoniae* and survival was assessed over the course of 4 days. A) Total RNA was extracted from whole, homogenized lungs and expression was determined via real time PCR. NLRP3 expression is dramatically increased 48 hours post *K. pneumoniae* infection. B and C) Mice lacking *Nlrp3* (B) or *Asc* C) demonstrated significantly increased mortality compared with wild type (WT) mice ($p < 0.05$, Logrank Test). D) No significant difference in survival was observed between WT mice and animals lacking Nlrc4 following *K. pneumoniae* challenge. Mock: n = 7; $Nlrp3^{-/-}$: n = 11; $Asc^{-/-}$: n = 4; $Nlrc4^{-/-}$: n = 7; WT: n = 19

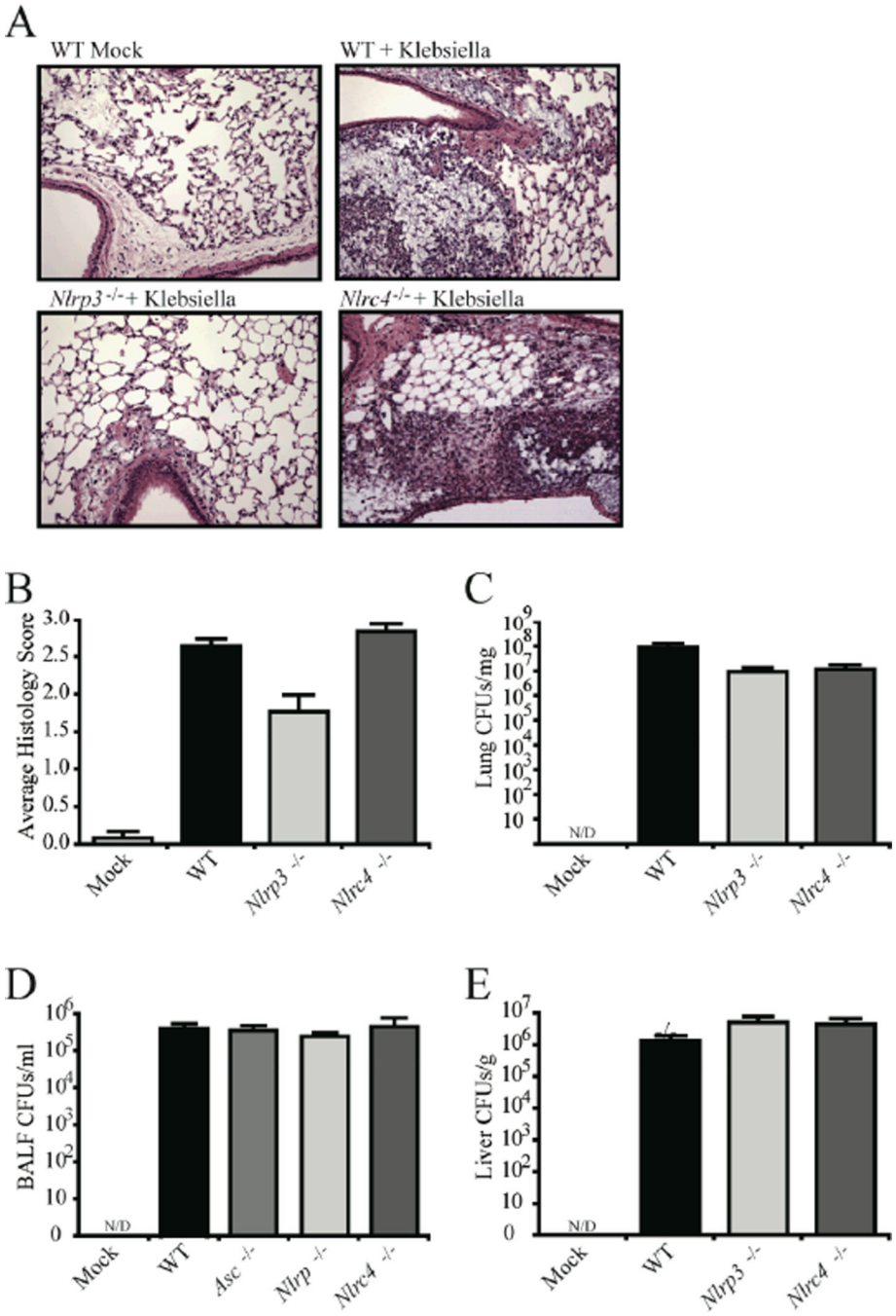

Figure 5. *Nlrp3* deficient mice demonstrated significantly attenuated airway inflammation following *Klebsiella pneumoniae* infection

A) In comparison to WT and *Nlrc4* deficient mice, mice lacking *Nlrp3* showed decreased inflammatory cell recruitment and less occlusion of the alveolar spaces following *K. pneumoniae* infection. Mice were challenged with 7.4×10^4 CFUs of *K. pneumoniae*. Whole lungs were harvested 48 hrs post-infection and each lobe was assessed and scored at specific locations along the main bronchi. Representative histology sections from the apical region of the main bronchi of the large lobe (10× magnification) are shown. B) Histology images were evaluated for a variety of inflammatory parameters and scored between 0 (absent) and 3 (severe). Significant attenuation in airway inflammation was observed in *K. pneumoniae*

challenged mice lacking *Nlrp3* $p < 0.05$). Mock, n=3; *Nlrp3*$^{-/-}$, n=6; *Nlrc4*$^{-/-}$, n=6; WT, n=12.

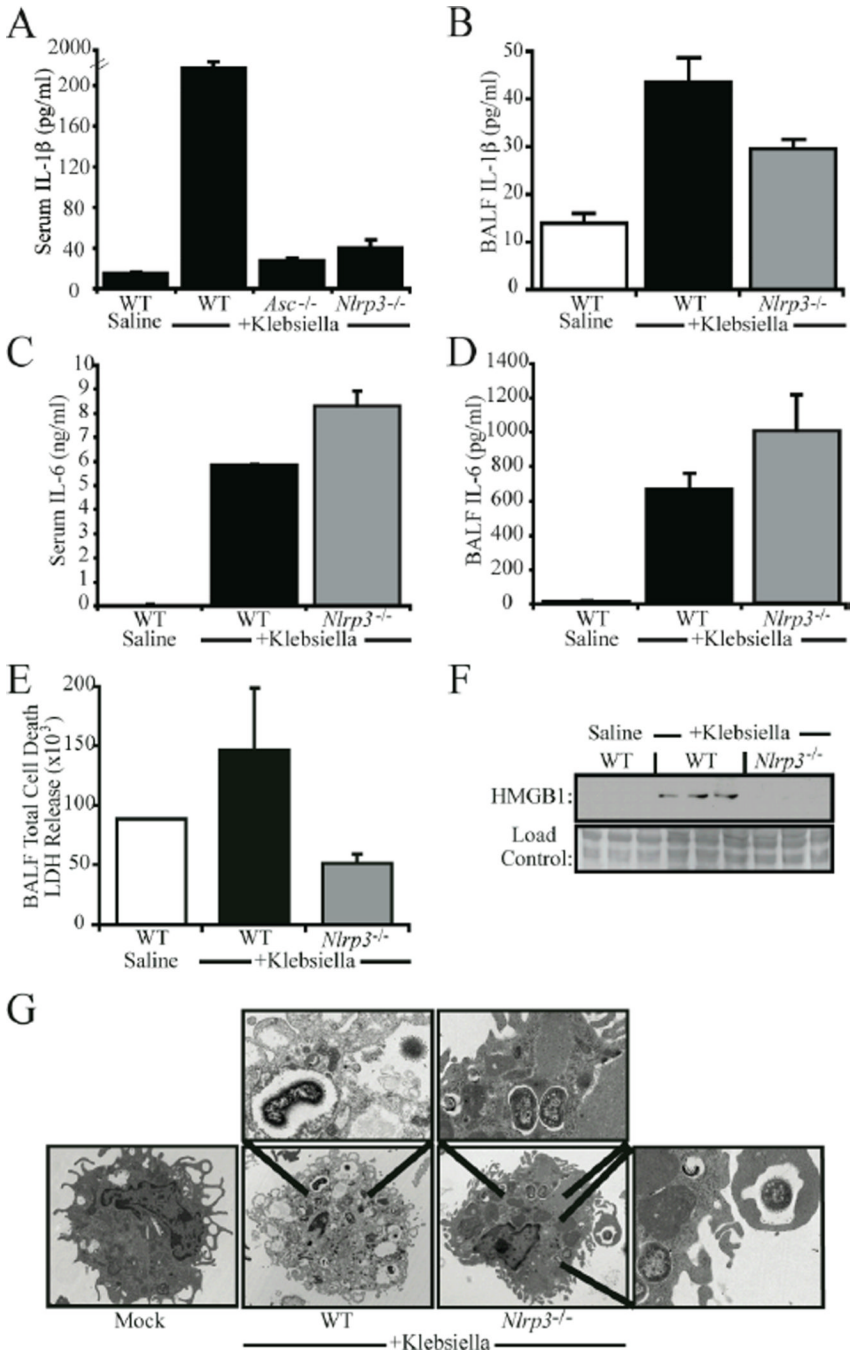

Figure 6. NRLP3 regulated *Klebsiella pneumoniae* induced IL-1β and necrotic cell death *in vivo*

A) Serum levels of IL-1β were significantly reduced in both *Nlrp3* and *Asc* deficient mice challenged with *K. pneumoniae* $p<0.05$). B) A modest, yet significant, decrease in *K. pneumoniae* induced IL-1β was observed in the BALF of *Nlrp3*$^{-/-}$ mice compared to WT ($p<0.05$). In contrast, IL-6 levels were elevated in the serum (C) and BALF (D) of *Nlrp3*$^{-/-}$ mice following *K pneumoniae* infection. IL-1β and IL-6 were measured by ELISA. E) Decreased levels of cell death were detected in the BALF of *K. pneumoniae* infected *Nlrp3* deficient mice as determined by LDH release. F) Serum levels of HMGB1 were dramatically reduced in mice lacking *Nlrp3* as determined by western blot analysis. G) Cells obtained by BALF were collected from *K. pneumoniae* challenged WT and *Nlrp3* deficient

animals and were subjected to electron microscopy analysis. Cells from WT mice exhibited cell death features morphologically consistent with necrosis. In contrast, cells obtained from *Nlrp3* deficient animals did not exhibit a morphology indicative of cell death. Magnified intracellular *K. pneumoniae* are shown in insets.