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The NLRP3 Inflammasome Mediates In Vivo Innate Immunity to Influenza A Virus through Recognition of Viral RNA

Irving C. Allen,[1] Margaret A. Scull,[2,3] Chris B. Moore,[1] Eda K. Holl,[2] Erin McElvania-TeKippe,[2] Debra J. Taxman,[2] Elizabeth H. Guthrie,[1] Raymond J. Pickles,[2,3] and Jenny P.-Y. Ting[1,2,*]
[1]Lineberger Comprehensive Cancer Center
[2]Department of Microbiology and Immunology
[3]Cystic Fibrosis/Pulmonary Research and Treatment Center
The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
*Correspondence: panyun@med.unc.edu
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SUMMARY

The nucleotide-binding domain and leucine-rich-repeat-containing (NLR) family of pattern-recognition molecules mediate host immunity to various pathogenic stimuli. However, in vivo evidence for the involvement of NLR proteins in viral sensing has not been widely investigated and remains controversial. As a test of the physiologic role of the NLR molecule NLRP3 during RNA viral infection, we explored the in vivo role of NLRP3 inflammasome components during influenza virus infection. Mice lacking Nlrp3, Pycard, or caspase-1, but not Nlrc4, exhibited dramatically increased mortality and a reduced immune response after exposure to the influenza virus. Utilizing analogs of dsRNA (poly(I:C)) and ssRNA (ssRNA40), we demonstrated that an NLRP3-mediated response could be activated by RNA species. Mechanistically, NLRP3 inflammasome activation by the influenza virus was dependent on lysosomal maturation and reactive oxygen species (ROS). Inhibition of ROS induction eliminated IL-1β production in animals during influenza infection. Together, these data place the NLRP3 inflammasome as an essential component in host defense against influenza infection through the sensing of viral RNA.

INTRODUCTION

Innate immune responses to influenza and other pathogens typically involve a highly conserved host-cell signaling mechanism designed to protect the host and get rid of harmful microbes. Recognition of conserved microbial structures, known as pathogen-associated molecular patterns (PAMPs) within cells of a myeloid origin is a hallmark feature of adequate host immunity. PAMPs are recognized by pattern-recognition receptors or sensors (PRRs), which subsequently initiate signaling cascades resulting in the production of proinflammatory cytokines and type-I interferons. Thus far, three families of PRRs, including the RLHs (RIG-I-like helicases), TLRs (toll-like receptors), and NLR (nucleotide-binding domain and leucine-rich-repeat-containing) proteins, have been identified and shown to be activated in response to viral pathogens (Akira et al., 2006; Ting et al., 2008). The individual members of these families can be distinguished by ligand specificity, cellular localization, and activation of unique downstream signaling pathways. The strategy of employing multiple families of PRRs affords the host a high degree of functional redundancy and provides multiple mechanisms to optimally respond to a diverse range of pathogens (Kawai and Akira, 2007).

The NLR proteins are emerging as a major route by which the innate immune system responds to microbial pathogens. Substantial evidence suggests that the NLR proteins serve as intracellular mediators of PAMP-initiated host-cell signaling, although the exact mechanisms underlying NLR responses to pathogens are not completely understood. One of the fundamental reactions of the innate immune response to viral infection is the processing and release of proinflammatory cytokines, including the regulation and release of IL-1β. It has been demonstrated that the NLR protein NLRP3, together with its adaptor protein, PYD- and CARD-domain-containing protein (PYCARD) (which is also known as Apoptotic Speck protein containing a CARD [ASC]), regulates IL-1β maturation through the formation of a biochemical complex called the inflammasome (Agostini et al., 2004). This inflammasome regulates the activation of caspase-1 and subsequent cleavage of the IL-1β and IL-18 precursors into their functional forms, which are then released from the cell. In addition to NLRP3, other NLR proteins, such as NLRC4 and NLRP1, also cause caspase-1 activation and IL-1β production (Mariathasan and Monack, 2007).

Although the function of the NLRP3 inflammasome in mediating responses to bacterial pathogens has been studied by several groups, the in vivo function of this important signaling complex during the course of viral infection is not well understood. In vitro transfection of DNA from viruses, bacteria, or mammalian sources into macrophages activates the NLRP3 inflammasome, but this result would suggest a global response to DNA rather than an antiviral response (Muruve et al., 2008). One report has shown that a synthetic analog of dsRNA, poly(I:C) activates IL-1β through the NLRP3 pathway (Kanneganti et al., 2006). However, another group failed to replicate this finding

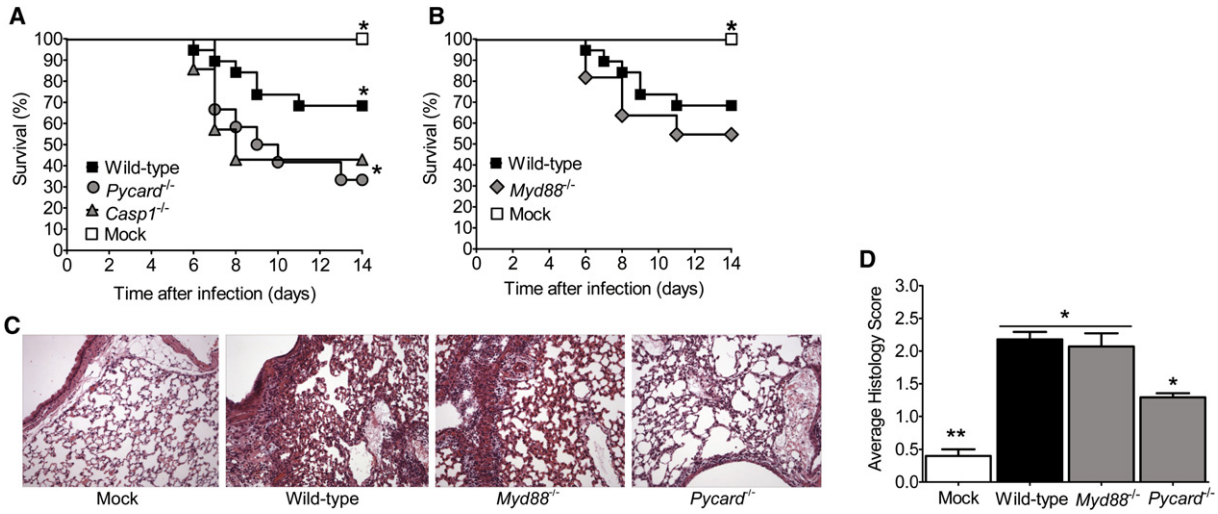

Figure 1. Characterization of Influenza Virus A/PR/8/34 Pathogenicity and Immune Response in Mice Deficient in Inflammasome Signaling Pathways

(A and B) The survival of *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice was measured and compared to wild-type (A) and *Myd88*$^{-/-}$ (B) mice (*$p < 0.05$; log rank). Mock-inoculated (n = 7); influenza-infected wild-type (n = 19); *Myd88*$^{-/-}$ (n = 11); *Pycard*$^{-/-}$ (n = 12); and *Casp1*$^{-/-}$ (n = 7).

(C) Lungs were harvested 3 days after infection. Sections through the main bronchiole of the left lobe were stained with H&E.

(D) Histological scoring of H&E-stained lung sections as shown in (C) (*$p < 0.05$; **$p < 0.01$; mean ± SEM). Wild-type (n = 8); *Myd88*$^{-/-}$ (n = 7); *Pycard*$^{-/-}$ (n = 6); and wild-type mock-infected (n = 4). Results are representative of at least two independent experiments.

(Muruve et al., 2008). Thus, the role of NLRP3 and the inflammasome during viral infection remains unresolved. Equally important, the majority of NLR characterization has been based on in vitro or ex vivo data generated from human cell lines and primary mouse cells. Although these studies have provided a wealth of information, the underlying relevance of these proteins in actual pathogenesis and host defense against pathogens in vivo has been much less defined.

To assess the contribution of NLR inflammasomes in viral pathogenesis, we assessed the host response to the influenza A virus. Influenza infection results in a highly contagious respiratory illness leading to substantial morbidity and occasionally death. Annual epidemics typically affect 5%–15% of the population and are thought to result in 250,000–500,000 deaths annually. Of the three types of influenza viruses, influenza A viruses are the most virulent to humans and are capable of infecting multiple mammalian and avian species. Human influenza A viruses can be further divided into different serotypeson the basis of the antibody response to the viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The influenza A virus genome is composed of eight individual strands of ssRNA, which encode a total of 11 different proteins.

In this report, we show that the NLRP3 inflammasome has a profound influence on in vivo host immune response and survival after airway infection with a mouse-adapted influenza A virus. Utilizing in vivo challenges with a synthetic analog of dsRNA, we demonstrated a possible mechanism underlying the NLRP3-associated innate immune response to viruses involved in the recognition of viral RNA. Furthermore, we extended the relevancy of these findings to human cells by establishing that ssRNA, dsRNA, and influenza A virus-mediated IL-1β release by human monocytes was dependent on the NLRP3 inflammasome and relied on intact lysosomal function, lysosomal enzymes such as cathepsin B, and reactive oxygen species (ROS). This report demonstrates that the NLRP3 inflammasome is an essential component of the in vivo host immune response to viral infection in a model system that is physiologically relevant to human disease.

RESULTS

Loss of NLR Inflammasome Activity Alters Survival and Inflammation in Response to the Influenza Virus

To assess the in vivo physiological contribution of NLR and TLR signaling pathways in response to influenza virus infection, we utilized gene-deletion mice in an influenza A/PR/8/34 virus infection model. Previous studies utilizing mouse primary macrophages have demonstrated that NLR components are necessary for IL-1β release in response to influenza virus infection in culture (Kanneganti et al., 2006). However, the in vivo physiological relevance of these findings has yet to be explored extensively. To assess the contribution of NLR inflammasomes in mouse survival and inflammation, we assessed mice deficient in either the NLR adaptor protein ASC (*Pycard*$^{-/-}$) or caspase-1 (*Casp1*$^{-/-}$). Mice lacking these inflammasome components showed significantly increased mortality after influenza infection (Figure 1A). As a comparison and as a control, we included the *Myd88*$^{-/-}$ mice. Previous studies have demonstrated that TLR3, 7, 8, and 9 represent the subset of TLRs that recognize viral nucleic acids and mediate the induction of type-I IFN (Kawai and Akira, 2007; Koyama et al., 2007). TLR7, 8, and 9 are dependent upon the TLR adaptor protein MyD88 for proper signal transduction. However, mouse survival was only moderately decreased for *Myd88*$^{-/-}$ animals, suggesting that removal of MyD88 and the disruption of the associated TLR7, 8, and 9

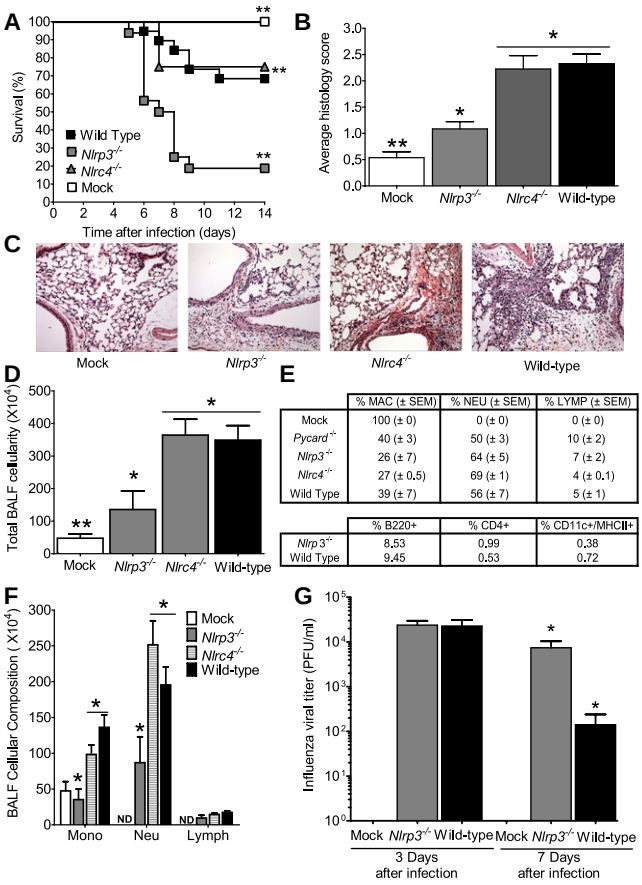

Figure 2. The Nlrp3 Inflammasome Is Required for Survival and Mediates Airway Inflammation and Viral Clearance after Pulmonary Challenge with Influenza Virus

(A) The survival of *Nlrp3*$^{-/-}$and *Nlrc4*$^{-/-}$ mice was measured and compared to that of the wild-type (**p < 0.01; log rank). Wild-type mock-infected (n = 7); wild-type infected (n = 19); *Nlrp3*$^{-/-}$ infected (n = 16); and *Nlrc4*$^{-/-}$ infected (n = 7). All survival experiments were performed together with those in Figure 1, therefore, the wild-type controls are identical to those shown in Figure 1.

(B) Histological scoring of H&E-stained lung sections as shown in (C) (*p < 0.05; **p < 0.01; mean ± SEM).

(C) Lungs were harvested 3 dpi. Sections through the main bronchiole of the left lobe were stained with H&E.

(D) Bronchoalveolar lavage (BAL) was performed, and the total number of cells present in the bronchoalveolar lavage fluid (BALF) was calculated (*p < 0.05; **p < 0.01; mean ± SEM).

(E) BALF cellular composition was determined via differential staining, and subpopulations were assessed via FACS. Wild-type influenza-infected (n = 8); *Nlrp3*$^{-/-}$ (n = 5); *Nlrc4*$^{-/-}$ (n = 3); *Pycard*$^{-/-}$ (n = 6); wild-type mock-infected (n = 4). Results are representative of three independent experiments.

(F) The number of monocytes, neutrophils, and lymphocytes was determined via differential staining of BALF cells (*p < 0.05; mean ± SEM). Wild-type influenza-infected (n = 8); *Nlrp3*$^{-/-}$ (n = 4); *Nlrc4*$^{-/-}$ (n = 4); wild-type mock-infected (n = 5).

(G) Influenza viral titers were determined by standard plaque assay either 3 or 7 dpi (*p < 0.05; mean ± SEM). Wild-type mock-infected, day 3 (n = 3); wild-type influenza-infected, day 3 (n = 9); *Nlrp3*$^{-/-}$ influenza-infected, day 3 (n = 9); wild-type mock-infected day 7 (n = 4); wild-type influenza-infected, day 7 (n = 6); and *Nlrp3*$^{-/-}$ influenza-infected, day 7 (n = 7).

signaling cascades only modestly affect mouse survival (Figure 1B).

To assess the extent of inflammation, we euthanized mice 3 days post-inoculation (dpi) and harvested the lungs for histology (Figure 1C). We observed a marked increase in airway inflammation, characterized by enhanced macrophage and neutrophil influx into the airways, 3 dpi. in wild-type and *Myd88*$^{-/-}$ mice as compared to the mock-inoculated wild-type. In contrast, the infected *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ (not shown) mice demonstrated substantially reduced airway inflammation. Histology scoring, by a blinded reviewer, confirmed the significant attenuation of airway inflammation in the *Pycard*$^{-/-}$ mice (Figure 1D). Together, these data demonstrate that both *Pycard* and *Casp1*, but not *Myd88*, are required for mouse survival and mediate airway inflammation in response to influenza virus infection.

The Nlrp3 Inflammasome Is Required for Mouse Survival and Inflammation after Influenza Infection

The above data suggest that proper activation of an NLR inflammasome is necessary for host survival and for mediating the innate immune response after influenza virus infection. It is well established that the NLR protein, NLRP3, can form an inflammasome complex with PYCARD and caspase-1 in response to a plethora of stimuli (Eisenbarth et al., 2008; Li et al., 2008; Martinon et al., 2006; Sutterwala et al., 2006). In addition to NLRP3, other NLRs such as NLRC4 have been suggested to form an NLRP3-independent inflammasome that functions in IL-1β maturation in response to *Salmonella typhimurium* and *Pseudomonas aeruginosa* infection (Mariathasan et al., 2004; Miao et al., 2008). After influenza inoculation, all animals demonstrated a decrease in body weight (Figure S1 in the Supplemental Data). However, as seen in Figure 2A, a dramatic decrease in survival was observed in *Nlrp3*$^{-/-}$ mice after virus infection, and no differences in mortality were observed between *Nlrc4*$^{-/-}$ and wild-type mice. Interestingly, the removal of Nlrp3 produced a more dramatic effect on animal survival than the removal of Pycard or caspase-1, suggesting the possibility that Nlrp3 might exert biologic effects in addition to caspase-1 activation; however, this remains to be explored.

As observed with the *Pycard*$^{-/-}$ animals, *Nlrp3*$^{-/-}$ mice show a significant decrease in airway inflammation after influenza infection. Histology scoring revealed no increase in inflammation in mock-infected animals, and only a modest increase was observed in the *Nlrp3*$^{-/-}$ mice, whereas a significant increase in airway inflammation was observed in the *Nlrc4*$^{-/-}$ and wild-type animals (Figure 2B). Histopathology assessments revealed a mild increase in airway inflammation in virus-infected *Nlrp3*$^{-/-}$ mice compared to mock-infected mice, but they revealed an exacerbated increase in *Nlrc4*$^{-/-}$ and wild-type animals 3 dpi (Figure 2C). To further characterize the composition of the cell populations infiltrating the airways during viral infection, we harvested bronchoalveolar lavage fluid (BALF) and assessed cellularity. Consistent with the histology findings, *Nlrp3*$^{-/-}$ animals demonstrated a significant decrease in total BALF cellularity when they were compared to either the *Nlrc4*$^{-/-}$ or wild-type animals (Figure 2D). Analysis of BALF cellularity indicates that macrophages and neutrophils represent the bulk of infiltrating cells in response to influenza. Differential profiling revealed no

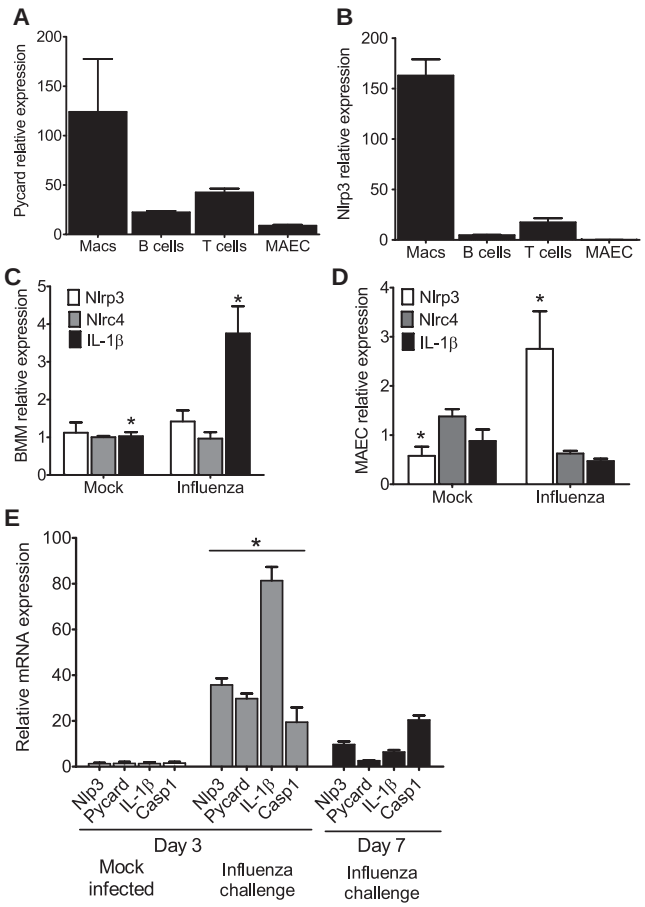

Figure 3. Nlrp3 Inflammasome Components Are Highly Expressed in Myeloid Cells and in the Lungs during Influenza A Virus Infection

(A and B) *Pycard* (A) and *Nlrp3* (B) gene expression was assessed in primary mouse bone-marrow-derived macrophages (Macs), B cells, T cells, mouse airway epithelial cells (MAECs), and mouse embryonic fibroblasts (MEFs) by RT-PCR; samples were normalized to 18 s and standardized to expression in MEFs. Each cell line was assessed in triplicate, and data are representative of three independent experiments.

(C and D) IL-1β, Nlrp3, and Nlrc4 transcripts were assessed by RT-PCR, normalized to 18 s, and standardized to naive levels after virus infection in primary mouse bone-marrow-derived macrophages (BMMs) (C) and MAECs (D) (*$p < 0.05$; mean ± SEM).

(E) Changes in mRNA expression of inflammasome components and IL-1β were assessed in the lungs over the course of influenza virus infection (*$p < 0.05$; mean ± SEM). All experiments are representative of at least two independent experiments with 3–5 mice per group.

significant differences in the percent cellular composition of the BALF between genotypes (Figure 2E); however, the *Nlrp3*$^{-/-}$ mice demonstrated an overall decrease in the number of monocytes and granulocytes (Figure 2F). Together, this indicates that the Nlrp3 inflammasome is an essential mediator of in vivo inflammatory responses to influenza virus.

We have observed that *Nlrp3*$^{-/-}$ animals show reduced immune responses after influenza challenge. To determine whether this reduction resulted in a defect in viral clearance, we harvested lungs from wild-type and *Nlrp3*$^{-/-}$ mice 3 and 7 dpi. During the early stages of infection (3 dpi), all influenza-challenged mice demonstrated an increase in viral titer, and no difference was observed between wild-type and *Nlrp3*$^{-/-}$ mice (Figure 2G). However, by 7 dpi. the *Nlrp3*$^{-/-}$ mice demonstrated approximately 2-fold higher viral titer in the lungs than did the wild-type animals (Figure 2G), providing evidence for a defect in viral clearance in the absence of Nlrp3.

Nlrp3 Inflammasome Components Are Expressed in the Lung and Myeloid Cells during Influenza Infection

To assess the relevance of Nlrp3 and Pycard in a panel of mouse cells, we assayed expression by quantitative RT-PCR in primary myeloid, lymphoid, and airway epithelial cells (Thompson et al., 2006; Willingham et al., 2007). Under naive conditions, both *Nlrp3* and *Pycard* genes were highly expressed in primary macrophages, and nominal expression was detected in T and B lymphocytes (Figures 3A and 3B). In the mouse airway epithelial cells (MAEC), only a low level of Pycard was detected, and Nlrp3 was not found (Figures 3A and 3B).

To characterize the effects of viral infection on gene transcription in these differentiated cell types, we challenged bone-marrow-derived macrophages (BMMs) and MAECs with influenza A/PR/8/34. No significant change in either Nlrp3 or Nlrc4 transcription was observed after viral challenge in the BMMs; however, a significant increase in IL-1β mRNA expression was detected (Figure 3C). In contrast, under naive conditions, Nlrp3 expression was low in the MAECs. After influenza infection, an increase in Nlrp3 transcription was observed, whereas no significant changes were found in Nlrc4 and Il-1β transcription (Figure 3D).

It should be noted that ex vivo cultured mouse BMMs were refractory to influenza virus infection, as shown by the precipitous drop in viral titer after exposure (Figure S2A). As a result of the lack of a productive infection, we often observed inconsistent IL-1β cytokine expression at the MOIs used in this study. For this reason, we did not pursue additional experiments with primary mouse macrophages. Even though IL-1β expression was inconsistent, at MOIs greater than 10 we were able to observe an increase in IFN-β at 12 hr, and we observed no differences between the genotypes (Figure S2B), indicating that the genes studied did not affect IFN-β production.

To further assess the contribution of the NLRP3 inflammasome in vivo, we sought to assess changes in mRNA expression of inflammasome components and IL-1β over a time course of influenza virus infection. Total lung RNA was extracted from tissue homogenates, and gene expression was assessed for Nlrp3, Pycard, Il-1β, and caspase-1 (Figure 3E). A significant increase in gene transcription was observed 3 dpi for all four assessed mRNAs. This was followed by a significant reduction of all mRNAs except caspase-1 by day 7 (Figure 3E). These results indicate that influenza induced a substantial increase in all components of the Nlrp3 inflammasome in the lungs and infiltrating cells of inoculated animals 3 dpi and also indicate that viral infection profoundly affects the inflammasome genes in a way that alters their biologic function.

To determine the in vivo contribution of the macrophages in the lungs of mice after virus infection, we harvested lungs 3 dpi and visualized cells containing viral antigen by IHC and confocal microscopy. The primary cells targeted in vivo in the mouse are the airway epithelial cells (Figures S3A–S3C), and 15% of macrophages are the second largest population (Figures S3D–S3F).

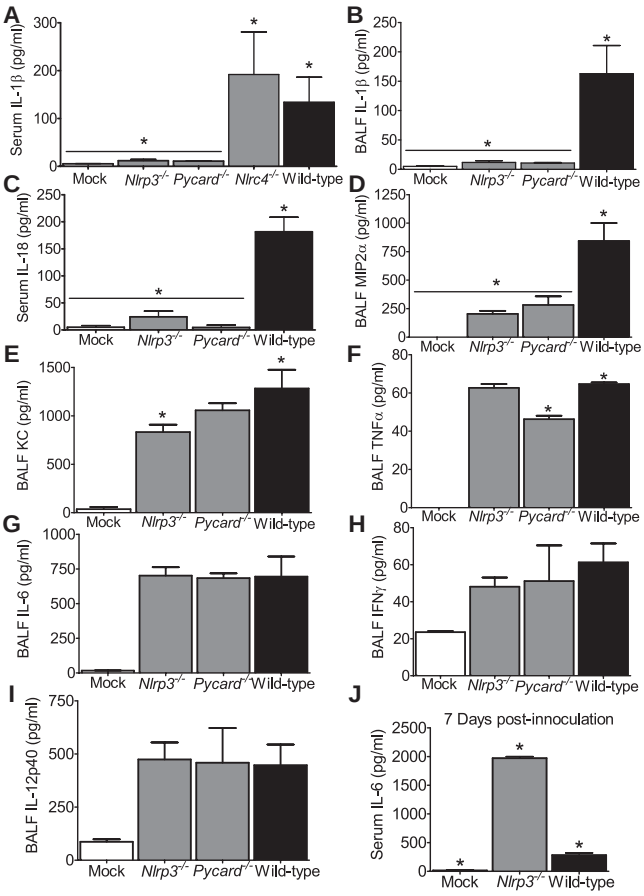

Figure 4. Mice Lacking Components of the Nlrp3 Inflammasome Demonstrate Significantly Altered Levels of Proinflammatory Cytokines after Pulmonary Challenge with Influenza

(A and B) IL-1β was assessed in serum (A) and BALF (B) from wild-type mock-infected, wild-type influenza-infected, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, and *Pycard*$^{-/-}$ mice, 3 days after infection.

(C–I) Serum levels of IL-18 were also determined in these animals (C). In addition to IL-1β and Il-18, MIP2α (D), KC (E), TNFα (F), IL-6 (G), IFNγ (H), and IL-12p40 (I) were also assessed (*$p < 0.05$; mean ± SEM). Mock, n = 5; *Nlrp3*$^{-/-}$, n = 6; *Nlrc4*$^{-/-}$, n = 6; *Pycard*$^{-/-}$, n = 9; wild-type, n = 5.

(J) Serum levels of IL-6 were determined 7 days after infection in wild-type mock-infected, wild-type influenza-infected, and moribund *Nlrp3*$^{-/-}$ mice (*$p < 0.05$; mean ± SEM). Mock, n = 3; *Nlrp3*$^{-/-}$, n = 6; wild-type, n = 3. All data are representative of at least two independent experiments.

However, a majority of macrophages and other leukocytes present in the lungs of infected mice were IHC negative (Figures S3G–S3I). Substantially smaller populations of polymorphonuclear cells and alveolar epithelial cells (<1%) were also infected with virus (not shown). Together, these data suggest that airway epithelial cells and macrophages are the likely cell types responsible for mediating the innate immune response during the initial stages of influenza virus infection.

NLRP3 Inflammasome Components Are Required for Influenza-Induced Proinflammatory Mediator Production

The above experiments indicate *Nlrp3*$^{-/-}$ and *Pycard*$^{-/-}$ mice demonstrate attenuated airway inflammation and reduced survival after influenza virus infection. We next sought to assess changes in serum and BALF cytokines known to play roles in host immune responses to viruses. A significant increase in IL-1β was detected in the serum and BALF from wild-type animals. This increase was dependent on Nlrp3 and Pycard but independent of Nlrc4 (Figures 4A and 4B). To demonstrate that this lack of IL-1β was due to reduced post-translational processing by the Nlrp3 inflammasome, we assessed lung transcription of *Il1b* and observed significant increases in IL-1β transcription in all mouse lines regardless of genotype (Figure S4). As previously mentioned, IL-18 has also been shown to be a critical mediator of influenza infection, and its maturation also relies on NLR inflammasome activation. A significant increase in serum IL-18 was detected in the wild-type mice (Figure 4C). However, as with IL-1β, IL-18 was significantly reduced to mock-infected amounts in mice lacking either Nlrp3 or Pycard (Figure 4C). KC, MIP2α, TNFα, IL-6, IFNγ, and IL-12, like IL-1β, facilitate a wide spectrum of immunologic functions during respiratory virus infection. To evaluate these cytokine concentrations in the lungs, we assessed the BALF supernatant by ELISA. A significant decrease in MIP2α was also observed in the *Nlrp3*$^{-/-}$ and *Pycard*$^{-/-}$ animals when they were compared to wild-type animals (Figure 4D), and these results correlated with the decreased cellularity observed in the BALF (Figure 2D). *Nlrp3*$^{-/-}$ mice also showed a modest decrease in KC (Figure 4E). *Pycard*$^{-/-}$ mice demonstrated a statistically significant decrease in TNFα, and such a decrease was not observed in the *Nlrp3*$^{-/-}$ animals (Figure 4F). The observed attenuation in TNFα in the *Pycard*$^{-/-}$ animals was observed in other assays and may reflect a previously-reported inflammasome-independent role for Pycard in the control of TNFα (Taxman et al., 2006). No significant differences were observed in IL-6, IFNγ, or IL-12p40 after influenza infection among the different mouse strains (Figures 4G–4I). Because of the increased mortality observed in the *Nlrp3*$^{-/-}$ mice, a subset of moribund animals was harvested 7 dpi. BALF IL-6 concentrations were significantly elevated in the *Nlrp3*$^{-/-}$ mice compared to the wild-type animals (Figure 4J). Although unexpected, this observation is consistent with clinical findings in humans, where influenza-infected individuals show increased IL-6 associated with increased morbidity (Lee et al., 2007).

NLRP3 Inflammasome Activation in Response to Influenza Is Dependent upon Lysosome Maturation and ROS

For clinical relevance, the above study of mice required verification in human cells. Airway epithelial cells are the predominant cell type infected by influenza, and previous studies have demonstrated that these cells contribute to innate immune responses (Kato and Schleimer, 2007). Primary human airway epithelial cell cultures (HAE) were infected with the human influenza virus A/Victoria /3/75 (H3N2), and the supernatant was harvested from either the apical or basolateral layer as previously described (Thompson et al., 2006). After infection, we observed robust viral replication in the HAE cultures (data not shown) and detected increased IL-1β concentrations in supernatant from the apical (Figure 5A) but not the basolateral layer. To expand upon these findings, we infected a human nasal airway epithelial cell line, JME, and observed increased *NLRP3* and

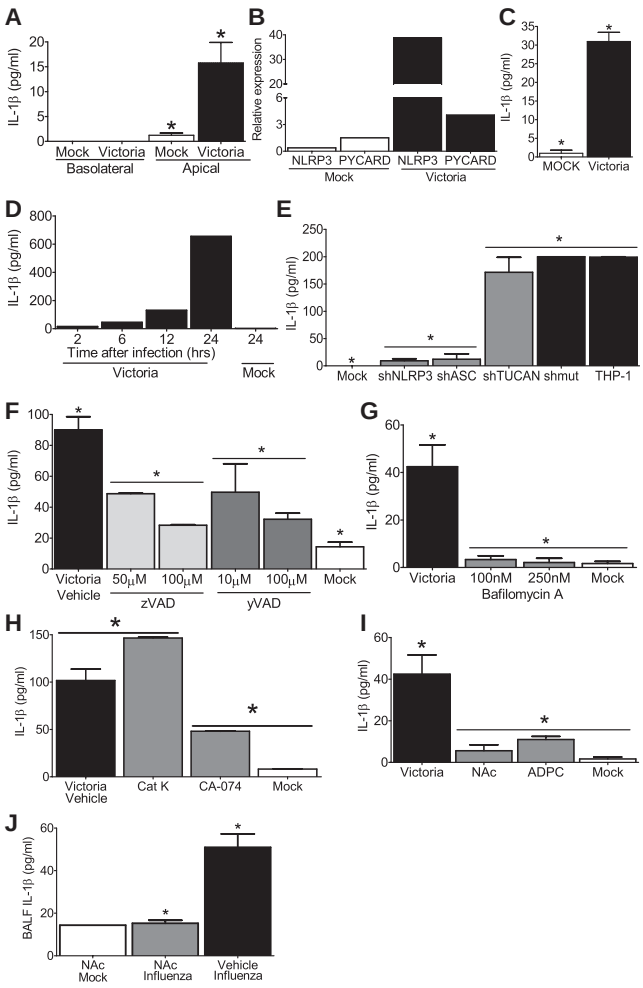

Figure 5. NLRP3 Inflammasome Activation in Response to the Influenza Virus Is Dependent upon Lysosomal Maturation and ROS Production in Human Cells

(A) IL-1β levels were determined in basolateral and apical washes from human airway epithelial cells challenged with influenza A/Victoria/3/75 (Victoria) 48 hr after infection (*$p < 0.05$; mean ± SEM).

(B) Human nasal airway epithelial cell lines (JME) were infected with A/Victoria/3/75, and changes in *NLRP3* and *PYCARD* mRNA expression were observed. Data were normalized to 18 s and compared to the expression in similarly treated human type II alveolar epithelial-like cell lines (A549).

(C) IL-1β was measured in the supernatants from A/Victoria/3/75-infected JME cells 24 hr after infection.

(D) Human THP-1 monocyte cell lines were infected with A/Victoria/3/75, and IL-1β was measured in the supernatant over a 24 hr time course.

(E) Human THP-1 cells were infected with lentivirus containing shRNA for *PYCARD* (shASC), *NLRP3* (shNLRP3) or *CARD8* (shTUCAN) or for a mutated sh target sequence (shmut). IL-1β was assessed in cell-free supernatants 24 hr after infection (*$p < 0.05$; mean ± SEM).

(F) The effect of ZVAD-CHO and Ac-YVAD-CHO on IL-1β release was determined in THP-1 cells after influenza infection (*$p < 0.05$; mean ± SEM).

(G–I) IL-1β release by THP-1 cells after influenza infection was also assessed after treatments with bafilomycin A (G), CA-074-Me (50 μM) and Cathepsin K Inhibitor I (Cat K) (10 μM) (H), and (2R, 4R)-4-aminopyrrolidine-2-4-dicarboxylate (APDC) (100 μM) and N-acetyl-L-cysteine (NAc)(50μM)(*$p < 0.05$; mean ± SEM) (I). All data are representative of at least two independent experiments.

(J) The in vivo effect of NAc (250 mg/kg) on BALF IL-1β after influenza infection was determined. (*$p < 0.05$; mean ± SEM). NAc mock, n = 1; NAc influenza, n = 5, vehicle Influenza, n = 3.

PYCARD expression (Figure 5B) and increased IL-1β release (Figure 5C). In addition to the JME cell lines, A/Victoria also induced a dramatic increase in IL-1β in a human monocytic cell line (THP-1) (Figure 5D). These data indicate that human cells of both monocytic and epithelial origin are contributors of IL-1β during influenza virus infection.

To verify that influenza-virus-mediated IL-1β production in THP-1 is dependent upon NLRP3 inflammasome components, we utilized small heteroduplex RNA (shRNA) knockdown to assess the contribution of NLRP3 and PYCARD. An shRNA against the third putative inflammasome component, TUCAN or CARDINAL (*CARD8*), was also targeted by shRNA. Successful RNA reduction is shown in Figures S5A–S5C. These cells were challenged with A/Victoria/3/75, and cell free supernatants were collected over a 24 hr time-course. No differences in viral titer were detected between the different knockdown and wild-type cell lines 24 hr after infection (Figure S5D). When cytokine was measured, a significant increase in IL-1β was observed in the wild-type THP-1 cells and cells transfected with a scrambled shRNA targeting sequence (shmut) 24 hr after infection, and this increase was significantly attenuated in cells containing shRNA targeting *PYCARD* and *NLRP3*, but not in those containing shRNA targeting *CARD8* (Figure 5E; Figure S5E). The role of *CARD8*, a gene found in humans but not mice, has not been previously studied by gene reduction. These data suggest that the CARD8 protein may not be important for NLRP3 inflammasome function induced by influenza virus. To confirm the specificity of shNLRP3 in IL-1β production, we also assessed TNFα release after influenza infection. As seen in Figure S5F, amounts of TNFα were not reduced in cell lines with shNLRP3 but were modestly reduced in cells with shASC, consistent with the mouse data described earlier. Finally, we demonstrate that ZVAD-CHO, a caspase inhibitor, and YVAD-CHO, a specific peptide inhibitor of caspase-1, both attenuated IL-1β amounts in a dose-dependent fashion after influenza virus infection (Figure 5F).

To further explore the mechanism underlying IL-1β release in response to influenza infection, we used pharmacological antagonist to block specific signaling pathways. Lysosomal degradation of particulate danger-associated molecular patterns (DAMPs) has been shown to activate the NLRP3 inflammasome (Hornung et al., 2008), but this has not been assessed in the context of viral infection. To test this, we utilized bafilomycin A to block lysosomal acidification via inhibition of the vacuolar H^+ ATPase system. The addition of bafilomycin A at concentrations used previously to inhibit DAMP signaling (Hornung et al., 2008) completely abolished influenza-induced IL-1β release (Figure 5G), which suggests a critical role for lysosomes in virus-mediated NLRP3 inflammasome function. A specific lysosomal cysteine proteinase, cathepsin B, has been associated with NLRP3-mediated cell death and IL-1β in response to nonviral signals (Willingham et al., 2007; Hornung et al., 2008). Utilizing the cathepsin B-specific inhibitor, Ca-074-Me, we observed a significant attenuation in IL-1β release after influenza challenge (Figure 5H). This suggests the importance of cathepsin B in NLRP3-mediated response to influenza virus but also suggests the involvement of additional lysosomal processes. This decrease was not observed with specific inhibitors for cathepsin K (Figure 5H). The release of lysosomal products

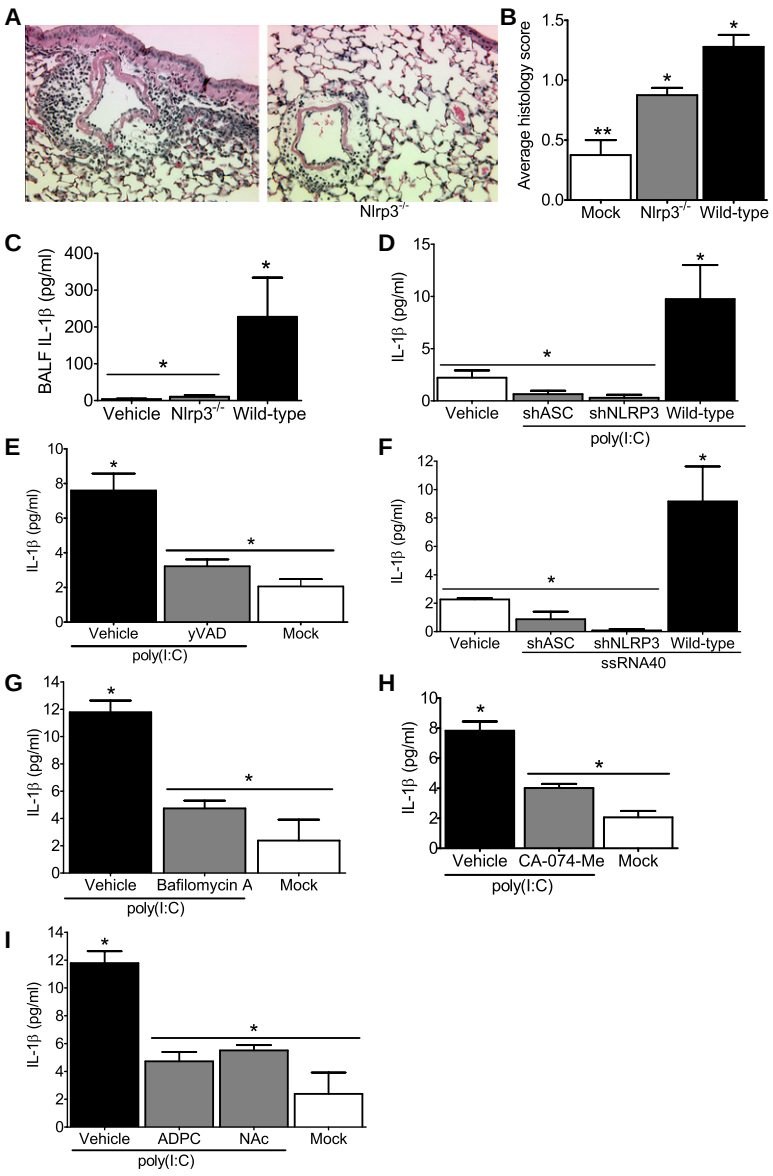

Figure 6. The NLRP3 Inflammasome Is Required for Airway Inflammation Induced by Nucleic Acid Analogs

(A) Lungs were harvested 24 hr after poly(I:C) challenge. Sections through the main bronchiole of the left lobe were stained with H&E.

(B) Histological scoring of H&E-stained lung sections as shown in (A) (*$p < 0.05$; **$p < 0.01$; mean ± SEM).

(C) BALF IL-1β was determined after poly(I:C) challenge in wild-type, *Nlrp3*$^{-/-}$, and mock-challenged wild-type mice (*$p < 0.05$; mean ± SEM).

(D) The effect of *PYCARD* (shASC) and *NLRP3* (shNLRP3) shRNA knockdown on IL-1β release in response to poly(I:C) in THP-1 cells was assessed (*$p < 0.05$; mean ± SEM).

(E) The effect of Ac-YVAD-CHO on IL-1β release was determined in THP-1 cells after poly(I:C) challenge (*$p < 0.05$; mean ± SEM).

(F) IL-1β secretion stimulated by ssRNA40 was assessed after the shRNA knockdown of *PYCARD* (shASC) and *NLRP3* (shNLRP3) (*$p < 0.05$; mean ± SEM).

(G–I) IL-1β release following poly(I:C) stimulation in THP-1 cells was assessed after treatments with bafilomycin A (100 nM) (G), CA-074-Me (50 μM) (H), and (2R, 4R)-4-aminopyrrolidine-2-4-dicarboxylate (APDC) (100 μM) and N-acetyl-L-cysteine (NAc) (50 μM) (*$p < 0.05$; mean ± SEM) (I).

into the cytosol has been demonstrated to promote the generation of ROS, and recent data have suggested that ROS generation by asbestos, monosodium urate and ATP is a necessary step in inflammasome activation (Dostert et al., 2008). To test whether this occurs during influenza virus infection, we treated THP-1 cells with the ROS inhibitors *N*-acetyl-$_L$-cysteine (NAc) or (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) (Dostert et al., 2008). Our data demonstrate attenuated IL-1β production from cells treated with either ROS inhibitor (Figure 5I), suggesting that ROS also contributes to NLRP3 inflammasome activation in response to influenza.

Influenza infection in vivo results in a substantial increase in ROS production in the airways of humans and mice. To assess the physiological relevance of the in vitro ROS inhibitor findings, we treated mice with the ROS inhibitor NAc via intranasal administration as previously described (Springer et al., 2007). Similar to the monocyte cell line findings, treatment with NAc completely inhibited the generation of IL-1β in the BALF after influenza infection (Figure 5J). In addition to NAc, bafilomycin A and CA-074-ME were also assessed; however, the pharmacokinetics for each of these compounds were not optimized for our in vivo assessments (data not shown). Collectively, these data suggest that NLRP3 inflammasome activation in response to the virus occurs when the cell senses alterations of lysosomal content and ROS induction in the cytosol after virus infection by using pathways similar to those activated by DAMPs.

Nlrp3 Mediates Airway Inflammation and IL-1β Induction by the Viral RNA Analogs poly(I:C) and ssRNA40

The data presented thus far demonstrate that Nlrp3 mediates in vivo airway inflammation in response to influenza virus infection in mice and inflammasome formation in both mice and humans. To further explore the mechanism underlying NLRP3 activation, we examine the in vivo immune response to the viral RNA analog, poly(I:C). To assess whether poly(I:C) is important for pulmonary inflammasome activation, mice were challenged intranasally with poly(I:C), and the resultant airway inflammation was characterized. Poly(I:C) induced moderate airway inflammation in the wild-type mice, and inflammation was significantly reduced in the *Nlrp3*$^{-/-}$ animals (Figure 6A). *Nlrp3*$^{-/-}$ mice exposed to poly(I:C) showed a significant reduction in the overall amount of airway inflammation (Figure 6B) and BALF IL-1β concentration (Figure 6C).

To verify this result in human cells, we utilized the shRNA knockdown cell lines described above and demonstrate that poly(I:C)-induced IL-1β is dependent on PYCARD and NLRP3 (Figure 6D). To confirm the role of caspase-1 in this mechanism, we exposed cells to the caspase-1 inhibitor yVAD-CHO, which significantly attenuated poly(I:C)-mediated IL-1β release

(Figure 6E). To determine whether the resultant NLRP3- and PYCARD-dependent IL-1β release was unique to poly(I:C), we also stimulated cells with single-stranded GU-rich RNA (ssRNA40) complexed with the cationic lipid LyoVec to facilitate uptake. A significant NLRP3- and PYCARD-dependent increase in IL-1β release was observed after ssRNA40 stimulation, indicating that NLRP3 and PYCARD mediate responses to ssRNA molecules (Figure 6F). To explore the intracellular pathways that lead to NLRP3 activation by RNA analogs, we utilized pharmacological inhibitors as described earlier and show that inflammasome activation in response to poly(I:C) requires lysosomal maturation, cathepsin B, and the generation of ROS (Figures 6G–6I). Together, these data suggest that NLRP3 inflammasome activation in response to virus is mediated by the recognition of viral RNA and involves pathways associated with lysosomal maturation and the induction of ROS.

DISCUSSION

In this report, we assess the in vivo contribution of NLRP3 to host immune responses to viral infection. NLRP3 forms a multiprotein inflammasome complex, which activates caspase-1 and leads to the maturation of several key proinflammatory cytokines, such as IL-1β and IL-18. It has been established that NLRP3 responds to both microbe-associated molecules and damage-associated molecular patterns (DAMPS) such as uric acid, alum salt, and silica (Dostert et al., 2008; Eisenbarth et al., 2008; Hornung et al., 2008; Li et al., 2008; Martinon et al., 2006). In all of these cases, it is clear that NLRP3 promotes inflammation. A key question is whether NLRP3 plays a beneficial or pathogenic role. In this study, we clearly demonstrate that NLRP3 and other components of the NLRP3 inflammasome play a beneficial role in the in vivo innate immune response to influenza infection and are required for normal host immune responses to the virus. An examination of the mechanism by which NLRP3 mediates host response to an RNA virus demonstrates that recognition of RNA analogs or RNA molecules is capable of mediating these responses. A further exploration of the mechanism shows that similar to the activation of NLRP3 by DAMPs such as particulate alum, MSU crystals and silica, activation of the NLRP3 pathway by the influenza virus, and more specifically by RNA analogs, is dependent on an intact lysosomal pathway and functional lysosomal enzymes such as cathepsin B and is also reliant on ROS both in vitro and in vivo. This supports the hypothesis that NLRP3 is activated through common intracellular changes caused by either PAMPs or DAMPs, rather than a model that evokes the direct interaction of NLRP3 with ligands of diverse molecular structures.

The recognition of viral pathogens by cells of the innate immune system is essential for the initiation of an ensuing inflammatory response. The endosomal TLRs TLR3, TLR7, and TLR8 and cytoplasmic RIG-I and MDA-5 are receptors of viral PAMPs and the primary route for the elicitation of host immune responses to viruses through the regulation of type-I interferons. Among the NLR family, there are few reports linking these proteins to the viral host response or viral pathogenesis. One report identifies endogenous NLRX1 as an inhibitor of the RIG-I and MDA-5 pathway through interactions with the essential mitochondrial antiviral signaling adaptor (MAVS) (Moore et al., 2008). However, the in vivo importance of NLRX1 remains to be determined. The involvement of NLRP3 in the macrophage response to RNA viruses has been controversial. One study has demonstrated the importance of Nlrp3 during viral infection in culture (Kanneganti et al., 2006), whereas another group was unable to confirm inflammasome activation after poly(I:C) challenge or infection with the RNA viruses reovirus and vesicular stomatitis virus in culture. Although we did not assess reovirus or VSV, our human monocytic cell line and in vivo mouse data clearly show that NLRP3 and PYCARD are indeed required for IL-1β maturation in response to influenza virus infection as well as to dsRNA analogs and ssRNA. Therefore, our data support the Kanneganti et al. study. However, we had difficulty studying influenza viral infection of mouse macrophages ex vivo because of a failure to establish a productive infection. Thus, we have not precisely duplicated the ex vivo data observed by the first report. A possible explanation that would reconcile the apparent discrepancies in the field is that different NLRs might mediate responses to different RNA viruses. The precedence for this is provided by MDA5 and RIG-I, which recognize different types of dsRNA and show viral specificity. RIG-I responds to RNA viruses, including paramyxoviruses and influenza virus, whereas MDA5 is essential for picornavirus recognition (Kato et al., 2008; Kato et al., 2006). Thus, it is possible that NLRP3 is responsible for dsRNA, ssRNA, DNA, Sendai virus, and influenza virus recognition and that an unidentified NLR is activated by reovirus and VSV.

Infection of mice with influenza virus results in a dramatic increase in morbidity and mortality and mimics many of the pathophysiological aspects of the human disease. The current study shows that Pycard and Nlrp3 deficiencies reduce inflammation in the lung, and this reduced inflammation is correlated with increased mortality and a viral clearance defect. Thus, the NLRP3 inflammasome-driven antiviral response is beneficial to the host after influenza infection. Although the precise role for both IL-1β and IL-18 in the immune response against viral pathogens remains elusive, in vivo assessments of IL-1R1- and IL-18-deficient mice have shown that these animals have reduced acute airway inflammation associated with influenza virus infection and that both have significantly decreased survival (Schmitz et al., 2005). These results are in agreement with our findings. Our data also highlight the observation that host response to influenza virus is specific and requires NLRP3 but not NLRC4. It should be noted that a recent study identified a role for ASC and caspase-1, but not Nlrp3, in the initiation of adaptive immunity against influenza virus (Ichinohe et al., 2009). Similar to the findings presented here, Ichinohe et al. demonstrate a role for both ASC and caspase-1 in mouse survival and in vivo host immune responses to influenza virus infection. Also similar to our findings, their data suggest that Nlrp3 is responsible for recognition of influenza virus within the alveolar macrophages and dendritic cells in a type I IFN-independent manner in culture. Additionally, we have not extensively assessed the establishment of CTL and IgA responses in our report; however, our work shows that NLRP3 did not affect IFN-γ production during viral infection and agrees with their observation that this gene does not affect adaptive immunity to influenza virus. However, Ichinohe et al. fail to establish a role for Nlrp3 in mouse survival and leukocyte recruitment to the

lungs, which represents a point of contention between this previously published work and the data we present here. One obvious difference between these publications is the subtle differences in the virus preparation, which may explain some of the opposing phenotypes.

Although our in vivo study shows that influenza virus is found in both airway epithelial cells and macrophages in the mouse, only mouse macrophages express detectable Nlrp3 transcript, and they are thus the likely cell type mediating the beneficial functions of host protection by Nlrp3. However, in humans we find NLRP3 expression in both airway epithelial cells and macrophages and provide further evidence that NLRP3 and PYCARD mediate inflammatory cytokine release in cells of human origin. Thus, NLRP3 and PYCARD may play an even more profound role in host inflammation and protection upon influenza infection of humans. Considering the disastrous nature of a possible influenza pandemic, our study suggests that targeting the NLRP3 inflammasome for enhanced function could become an important therapeutic measure against such an infection.

EXPERIMENTAL PROCEDURES

Experimental Animals

All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee guidelines of University of North Carolina, Chapel Hill. The generation of mice lacking functional *Nlrp3* (Cryopyrin), *Nlrc4* (ICE-Protease Activating Factor), Apoptotic Speck protein containing a Card (*Pycard*), *Casp1*, and *Myd88* has been previously described (Adachi et al., 1998; Mariathasan et al., 2004; Sutterwala et al., 2006). The *Nlrp3*$^{-/-}$ mice were originally produced at Millenium Pharmceuticals, were kindly provided by Dr. Richard Flavell as N5, and were backcrossed for another four generations at UNC-CH, resulting in mice that were backcrossed for a total of nine generations.

Virus Propagation

Influenza virus A/PR/8/34 (H1N1) was propagated in the allantoic cavity of 10-day-old embryonated specific-pathogen-free chicken eggs and was mouse adapted by a minimum of six serial passages through mice as previously described (Cottey et al., 2001). The influenza virus A/Victoria/3/75 (H3N2) is a recombinant virus generated from cloned cDNA in 293T cells and propagated in MDCK cells. Viral titers were determined by standard plaque assay on confluent monolayers of MDCK cells.

Influenza Virus Infection of Human Primary Cells and Cell Lines

Cells were challenged with A/Victoria (MOI = 1) for 2 hr at 37°C. After incubation, supernatant was replaced with fresh media, and cells were incubated at 37°C. Cell-free supernatants were harvested at select time points for viral titer and cytokine analysis. To assess viral PAMPs, we incubated cells for 24 hr in the presence of naked poly(I:C) (10 μg)(Sigma) or ssRNA40 complexed with LyoVec (InvivoGen) (10 μg). For pharmacological assessments, cells were treated with bafilomycin D (100–250 nM), Ac-ZVAD-CHO (10–100 μM), Ac-ZVAD-CHO (10–100 μM), Ca-074-Me (10–50 μM), Cathepsin K Inhibitor I (10–50 μM), N-acetyl-L-cysteine (NAC) (50–100 μM), or (2R, 4R)-4-aminopyrrolidine-2-4-dicarboxylate (APDC) (50–100 μM) as previously described (Dostert et al., 2008; Hornung et al., 2008; Willingham et al., 2007).

In Vivo Influenza A Virus Infection and poly(I:C) Challenge

Animals were anesthetized and challenged by intranasal administration of 6×10^4 PFU/ml of influenza virus A/PR/8/34 in 50 μl of PBS and 0.1% alum (Sigma). Mice were observed and weight was assessed daily for up to 14 dpi. For assessment of poly(I:C) in vivo, wild-type and NLRP3$^{-/-}$ mice received two doses (50 μg/dose) of poly(I:C) in 50 μl of PBS and 0.1% alum or vehicle on alternating days and were harvested 24 hr after the second administration. For assessment of in vivo ROS production, mice were treated with the ROS inhibitor NAc (250 mg/kg) via intranasal administration (as previously described by Springer et al., 2007) throughout the course of virus challenge.

Influenza-inoculated mice were euthanized either 3 or 7 dpi, and serum was collected after cardiac puncture. Total cytokine levels were determined by ELISA (R&D Biosystems or BD Biosciences). In some cases, bronchoalveolar lavage (BAL) was performed, and the number of cells present in the BAL fluid (BALF) was determined with a hemacytometer. A morphology-based differential cell count was conducted on cytospin preparations from the BALF and stained with Diff-Quik solution (Sigma). BALF cellularity was also determined via FACS with antibodies reactive to CD11b, CD11c, GR-1, B220, CD4, and CD8 according to standard techniques. Centrifuging the remaining BALF removed cells, and cytokine levels in the supernatant were determined.

For histopathologic examination, lungs were fixed by inflation and immersion in 10% buffered formalin. To evaluate airway inflammation, we subjected fixed lung slices to hematoxylin and eosin (H&E) staining. Evaluators who were blinded to genotype and treatment scored lung sections (0 [none]–3 [extreme]) on the basis mononuclear and polymorphonuclear cell infiltration, perivascular and peribronchiolar cuffing, and estimates of the percent of lung involved with the inflammation.

For assessment of viral titer, whole lungs were removed, weighed, and homogenized in PBS. Lung viral titers were determined by standard plaque assay in the supernatants.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) followed by Tukey-Kramer HSD for multiple comparisons was performed on complex data sets. Statistical significance for single data points was assessed by the Student's two-tailed t test. Survival curves were generated via the product-limit method of Kaplan and Meier, and comparisons were made via the log rank test. In all cases, a p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and five figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00139-3.

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